DIFFERENTIATING HUMAN AND NONHUMAN SOURCES OF *ESCHERICHIA COLI* BY PHENOTYPIC AND GENOTYPIC METHODS

By

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Dedicated to my parents with regards
who are no longer in this world

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DIFFERENTIATING HUMAN AND NONHUMAN SOURCES OF *ESCHERICHIA COLI* BY PHENOTYPIC AND GENOTYPIC METHODS

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Estuarine waters receive fecal pollution from a variety of sources including human and wildlife. *Escherichia coli* is one of several fecal coliform bacteria that inhabit the intestine of many warm-blooded animals. Methods are required to specifically differentiate sources of pollution, which impact estuaries and influence remediation efforts.

E. coli were isolated from human and nonhuman sources in the Apalachicola National Estuarine Research Reserve (ANERR) and analyzed for multiple antibiotic resistance (MAR), fatty acid methyl ester (FAME), O-serogroups, ribotype (RT), pulsed field gel electrophoresis (PFGE) and plasmid profiles. *E. coli* from human sources showed significantly ($P < 0.05$) greater resistance to antibiotics and higher MAR indices than isolates from nonhuman

sources, except for penicillin G. Sixty-five different patterns were observed among human source isolates, compared to 32 for nonhuman source isolates. Profiles homology based on coefficient similarities showed that human source isolates were more diverse than nonhuman source isolates. All isolates analyzed for FAME profiles contained large amounts of hexadecanoate, cis-7 hexadecenoate, cycloheptadecanoate and summed feature 7. Human and nonhuman source isolates showed 19 and 26 O serogroups, respectively, and the predominant O serogroups of human source isolates differed significantly ($P<0.001$) from those of nonhuman source isolates.

Ribotype and PFGE studies showed that human source isolates were less diverse in profiles than nonhuman source isolates. Plasmid analysis showed that there was no association between MAR pattern and plasmid profile.

FAME and PFGE profiles did not correlate with source, however, MAR, O serogroups and RT profiles did. It was also found that MAR, FAME, O serogroup, and RT profiles did not correlate with each other. However, identical RT profiles often exhibited the different MAR and O serogroup, indicating that *E. coli* populations originate from a variety of sources and may undergo phenotypic change in the aquatic environments. *E. coli* obtained directly from human and animal feces had high similarity with human and nonhuman source isolates, respectively.

Validation of MAR and RT in a separate estuary showed that MAR and RT profiles of *E. coli* may offer useful methods to identify sources of fecal pollution within estuaries and to facilitate management practices.

INTRODUCTION

Fecal pollution is a major problem in almost all estuaries (29). This pollution can originate from human (HS) and nonhuman sources (NHS). The economic consequences of fecal pollution are particularly significant for fishery resources, especially shellfish industries where harvest areas are strictly defined by levels of fecal pollution. Understanding sources of fecal pollution assists management of waters where seafoods are harvested. Although much effort has been given to define indicators of fecal pollution, there are insufficient methods which differentiate HS and NHS of fecal pollution.

The Apalachicola National Estuarine Research Reserve (ANERR) is a unique environment to study HS and NHS of fecal pollution. The Reserve encompasses more than 193,000 acres of land constituting two barrier islands, the lower 20 miles of the Apalachicola River and its floodplain, adjoining upland and wetlands, and the Apalachicola Bay system. In the ANERR, extensive protected upland and wetlands are the major habitat of local wildlife. The ANERR is also a significant harvest area for shellfish and a variety of finfish. Oysters are the most important commercial invertebrate in the Bay system (47, 109).

The Rookery Bay National Estuarine Research Reserve (RBNERR) is a large preserve encompassing 12,500 acres, managed by the Florida Department of

Environmental Protection It is located at the northern end of the 10 000 islands on the Gulf coast of Florida and represents one of the few remaining undisturbed mangrove estuaries in North America It contains HS and NHS of fecal pollution analogous to study areas in the ANERR A principal HS of pollution is Henderson creek which bisects the reserve The reserve contains diverse potential sources of nonhuman pollution originating from wildlife area, as well as aquatic life including shellfish, numerous species of finfish, manatees and dolphins (109) For this reason, effective management of ANERR and RBNERR resources requires tools to identify sources of fecal pollution that degrade water quality, close shellfish beds, thereby assisting in better management practices

Several attempts have been taken to develop methods to differentiate the sources of fecal pollution Initially the ratio of fecal coliform to fecal streptococci was used as an indicator of the sources A ratio of 4 or more was considered to indicate a human source, while a ratio of 0 7 or less suggested an animal source (58) This ratio has since proven unreliable, and the method has been abandoned (126) *Bacteroides fragilis* has been proposed as an indicator of fecal pollution However the survival of *B. fragilis* in water is lower than that of *E. coli* or *Streptococcus faecalis* Moreover it can not differentiate HS and NHS of fecal pollution (53, 80) Bacteriophages have also been proposed as indicators of the source of fecal pollution Several investigators observed that animal and human feces contained different serotypes of RNA coliphages (54, 118). The usefulness of these methods is limited because only a small percentage of fecal samples contained the phages (118).

The fecal coliform, *E. coli*, has been used as an indicator of human enteric pathogens for many years (56). However, it is well established that *E. coli* also inhabit the intestines of other warm blooded animals. Human feces potentially carries many enteric pathogens such as *Salmonella typhi*, *Salmonella paratyphi*, *Shigella* spp., pathogenic forms of *E. coli*, Hepatitis A, and Norwalk-group viruses. In contrast, animals are usually resistant to human viruses, and as such rarely carry these in their intestine (72, 93, 144). Conversely, most cases of salmonellosis caused by species other than *S. typhi* and *S. paratyphi* are of animal origin (118). Therefore, before *E. coli* can be used to indicate the risk of water, it is important to know if pollution originates from humans or animals. Consequently, research is needed to determine potential characteristics of *E. coli* that can be used to identify its source from various inputs of fecal pollution. In this manner, more accurate health risks can be assessed and remediation efforts can be enhanced.

Bacterial pathogens have been typed and differentiated by biochemical tests (2, 113), phage susceptibility (159), outer membrane protein profiles (18), antibody reactivity (154), fimbriation (94), bacteriocin production and susceptibility, and other methods (65). However, these systems have disadvantages including 1) unstable phenotypes, 2) low sensitivity at the intra-species level, and 3) limited specificity (145).

Recent techniques of DNA analysis have reduced dependence on phenotypic traits. These methods, such as plasmid profile analysis, comparison of DNA restriction endonuclease digestion fragment patterns, and the use of nucleic acid probes, have been used to discriminate bacterial strains within a single species (145). Analysis of chromosomal DNA avoids the potential pitfalls associated with variable expression of phenotypes and with the biologic instability of plasmids. It is possible that these techniques can be used to discriminate sources of *E. coli*.

It is well known that differentiation of bacterial strains by genotypic traits is more reliable than phenotypic traits. However, phenotypic traits such as multiple antibiotic resistance (MAR), fatty acid methyl ester (FAME), and serotype have been extensively used to type bacterial strains (39, 86, 93 107).

Multiple antibiotic resistance (MAR) is a method which has been used to differentiate *E. coli* from different sources using antibiotics commonly associated with human and animal therapy, as well as animal feed (88, 93, 122, 123).

Gas liquid chromatography of bacterial cellular fatty acid methyl esters (FAME) has been used in clinical microbiology as either a primary or adjunctive means for identification of many medically important bacteria (91, 107). It has been well established that fatty acid composition of a microorganism is an important taxonomic character and that fatty acid can be analyzed quantitatively to provide useful taxonomic information at the species level and in some cases sub species (91, 107, 153).

Serotyping microorganisms is based on the presence or absence of somatic (O), flagellar (H), capsular or envelope (K) antigenic determinants and their reaction with specific antisera. Several investigators have used this method for discriminating *E. coli* from different sources (35, 63).

Ribotyping, a method of "DNA fingerprinting," has provided a very powerful tool to differentiate and cluster bacterial strains (25, 90, 94, 145, 149). Ribosomal RNA (rRNA) is ubiquitous, highly conserved, and multiple copies are present on the bacterial chromosome. These factors have allowed use of a single probe to characterize phylogenetically distant bacteria following digestion of DNA with restriction endonucleases. Generally, members of *Enterobacteriaceae* yield more restriction fragments than other bacteria, due to greater number of rRNA operons (70).

Pulsed field gel electrophoresis (PFGE) is another measurement of restriction fragment length polymorphism (RFLP) that highly resolves bacterial strains. Numerous investigators have described its use in differentiating bacteria from specific sources, especially in epidemiological studies (9, 119, 127, 132). This technique involves digestion of chromosomal DNA by low frequency restriction endonucleases to produce high molecular weight fragments. DNA fragments are then separated on a polymeric gel by alternating pulsed-electric fields.

Plasmids are extrachromosomal, double-stranded autonomously replicating circular DNA molecules. Phenotypic properties of plasmids include antibiotic resistance, enterotoxins and colicins production (15, 45, 104). The application of plasmid and restriction endonuclease analyses of plasmid DNA can be a promising means to identify strains within a given species (15, 26, 104). The underlying principle is that isolates of the same strain contain the same number of plasmids with the same molecular weights and restriction digest patterns. In contrast, epidemiologically unrelated isolates have distinctly different plasmid "fingerprints" (104). Antibiotic resistance patterns and their association with plasmid profiles of *E. coli* have been described by numerous investigators (96, 103, 104, 114).

The overall goal of this dissertation was to test the hypothesis that phenotypic and genotypic properties of *E. coli* can differentiate HS and NHS of fecal pollution. The specific objectives were as follows:

1) To isolate and identify *E. coli* from HS and NHS,
2) To analyze MAR, FAME, O-serogroup, RT, and PFGE profiles of *E. coli*,
3) To correlate MAR, FAME, O-serogroup, RT, and PFGE profiles of *E. coli* with HS and NHS of fecal pollution,
4) To determine association between MAR and plasmid profiles, and
5) To compare MAR and RT profiles of HS and NHS of *E. coli* between the reserves.

REVIEW OF LITERATURE

Escherichia coli

The organism now known as *Escherichia coli* was first isolated and described by Theodor Escherich in 1885 under the name *Bacterium coli commune* from infant feces. It belongs to the family *Enterobacteriaceae*. There are several other species in the genus *Escherichia*, the best known is *E. coli*, which has a variety of serotypes. Although *E. coli* is a normal inhabitant of the intestinal tract of man and animals, specific pathogenic forms are associated with different types of intestinal and extra intestinal infections. It can also be used as an indicator of fecal contamination (79-81, 92, 146).

General Characteristics

E. coli is gram-negative, asporogenous, facultatively anaerobic, non-acid-fast, motile by peritrichous flagella or non motile, fimbriated and straight rod, 1.1-1.5 x 2.0-6.0 μm occurring singly or in pairs. A capsule or microcapsule is often present and few strains produce a polysaccharide slime layer (81, 92).

It grows readily on simple culture and synthetic media with glycerol or glucose as the sole source of carbon and energy. When growing anaerobically there is an absolute requirement for fermentable carbohydrate. Glucose and other carbohydrates are fermented to produce acid and gas. Lactose is fermented by most strains but fermentation may be delayed or absent. It is catalase-positive, oxidase-negative, indole-positive, methyl red-positive, Voges Proskauer negative, usually citrate-negative, negative for H_2S, urea hydrolysis, and lipase (81).

In liquid culture media, smooth (S) strains of *E. coli* produce uniform turbidity, while rough strains (R) tend to leave a clear supernatant medium over a granular deposit of growth. The generation time of most strains is 20-30 min. On solid media, colonies are circular and smooth and some strains produce mucoid colonies (101). On media containing washed erythrocytes, a soluble α hemolysin can be demonstrated. There is a cell associated β-hemolysin which is released from cells by lysis (136).

Strains show a wide range of growth temperature, from 5-45°C, with an optimum of 37°C. The minimum a_w for growth is 0.94 in the pH range 4.3-10, with pH 6.8 as the average. The genome size is between 2.3×10^9 and 3.0×10^9 daltons and G+C content is 48 to 52% (81, 82, 146).

Distribution

The primary habitat of *E. coli* is the lower intestinal tract of warm-blooded animals. Colonization occurs shortly after birth and its source is to be found in the mother and the inanimate environment (111). At any one time, most normal individuals carry several strains of *E. coli* in their intestinal tract, including a small number of resident clones exhibiting a rate of replacement measured in weeks or months, and a much larger number of transient clones that are replaced in a matter of days or weeks. The overall density of *E. coli* in the mammalian colon is approximately 10^8 cells/gram of colon contents. The secondary habitats of *E. coli* are soil, sediment, and water, usually as a result of fecal contamination, where its half life is thought to be only a few days (73, 137, 146).

Methods for Isolation and Identification

Methods used for isolation and identification of *E. coli* depend on the type of sample and the purpose of study. Anon (12) described the standard multiple tube test for enumeration of *E. coli* in water. He used minerals modified glutamate medium at 37°C as a first selective step, with lauryl sulphate lactose broth as an alternative, subcultured the gas producing tubes in brilliant green bile broth (BGLB) or lauryl sulphate tryptose broth at 44°C, and confirmed *E. coli* by the indole test. A rapid alternative is the single tube confirmation test based upon formation of gas from mannitol, and indole from tryptophan, according to Pugsley et al. (128).

The American Standard Methods for the Examination of Water and Waste Water (9) describe the standard most probable number (MPN), presence-absence test and membrane filter enumeration methods both for total and fecal coliforms, but not for *E. coli*. However, it is recommended that detection of *E. coli*, if wanted, can be performed by the IMViC test (79).

Usually, large numbers of *E. coli* are expected in feces and sewage treatment plant effluents. In these cases, samples can be plated directly on

MacConkey agar or eosin methylene blue (EMB) agar, while often a sample is enriched overnight in tryptic soy broth (TSB) or brain heart infusion (BHI) and samples from TSB or BHI are plated on MacConkey or EMB agar the next day. Identification can be performed by IMViC test, or by using commercially available identification systems (API, Enterotube, etc.) (79).

Fluorogenic detection and enumeration methods for *E. coli* in water and other samples have been reported (129). These assays are based on the activity of β-glucuronidase which cleaves the substrate 4-methylumbelliferyl-β-D glucuronide (MUG) into 4-methylumbelliferyl moiety which produce a blue fluorescence when irradiated with long wave ultraviolet (UV) light. Because β-glucuronidase activity is not fully specific for *E. coli*, it should be confirmed by biochemical tests. Another problem is that most enterohemorrhagic *E. coli* (EHEC) isolated are β-glucuronidase negative (44), which interferes with their detection. Detection based on MUG identification should not, therefore, be the method of choice when the presence of EHEC strains is suspected. MUG can also be added to selective media, e.g. lauryl sulphate tryptone broth (LTB). After incubation, fluorescence can be examined by direct irradiation of the culture with long wave UV light. MPN tests using MUG added to LTB (LTB-MUG) and plate counts using violet red bile agar containing MUG (VRB-MUG), were developed by Feng and Hartman (52). Other presence-absence test for total coliforms (TC) and *E. coli* in drinking water detect β-galactosidase and β-glucuronidase activity. Commercial versions of this approach include Colilert, Colisure, and Fluorocult. These use visual detection of color and fluorescence and require 14 to 18 h to complete (6, 152).

Hernandez et al. (78) developed a microtitration plate MPN (mMPN) to detect *E. coli* in marine and estuarine waters. They used one third of the plate for per dilution and detected *E. coli* with MUG. It has been found that the new MPN is an alternative to standard 3- and 5-tubes MPN tests because of the increased precision, savings in time, space, media and cost.

A new membrane filter agar medium (MI agar) containing a chromogen indoxyl ß-D-glucuronide and a fluorogen, 4-methylumbelliferyl-ß-D galactopyranoside, was developed to detect and enumerate *E. coli* and total coliforms (TC) in water samples on the basis of enzymatic activity. TC produce ß-galactosidase, which cleaves 4-methylumbelliferyl-ß-D galactopyranoside to form 4-methylumbelliferone that fluoresced under long wave UV light. *E. coli* produce ß-glucuronidase, which cleaves indoxyl ß-D-glucuronide to form a blue color. The recovery of TC and *E. coli* from the new medium was compared with those of mEndo agar and two *E. coli* media, mTEC agar and nutrient agar supplemented with 4-methyumbelliferyl-ß-D glucuronide, using natural and spiked water samples. On average, the new medium recovered 1.8 times as many TC as mEndo agar, with greatly reduced back ground counts. MI also recovered more *E. coli* than nutrient agar supplemented with 4-methylumbelliferyl-ß-D glucuronide (30). Therefore MUG can be considered as a reliable immediate confirmation test for *E. coli* and reagents for this test are commercially available. In a combination with membrane filtration, Farber (49) used the MUG test as the final confirmation step in the enumeration of *E. coli* in food by direct plating.

Recently, significant progress has been made in rapid detection of *E. coli* using modern membrane filter techniques. These membrane filter methods, in principle, can be used without limitations in all disciplines of microbiology, both for enumeration of bacteria as marker organisms and for identification of bacteria with specific traits such as virulence factors. The introduction of new confirmation tests such as MUG hydrolysis has contributed in this respect. Furthermore, the combination of membrane filter methods with monoclonal antibody or DNA hybridization allow a rapid confirmation of pathogenic *E. coli* strains due to availability of monoclonal antibody and DNA probes specific for LT, ST, and verotoxins (73).

Multiple Antibiotic Resistance (MAR)

Due to the use of antibiotics in human and animals, increased antibiotic resistance occurs. Different bacterial clones can be characterized by their antibiotic resistance pattern. Specific pattern may be helpful in epidemiological investigation of strains. It is important, of course, to remember that resistance characters are transmissible and by nature not stable (95). However, several attempts have been made to compare the patterns of antibiotic resistance in *E. coli* with the sources of isolates. To assess the association of resistance with source Kaspar et al. (86) isolated a total of 202 *E. coli* from rural and urban waters and tested them with 11 antibiotics commonly associated with animal faecal and/or clinical treatments. They found that 90% and 32% of urban and rural *E. coli* isolates, respectively, were resistant to one or more antibiotic, and the average MAR index for urban areas was 0.090, 3.2 times greater than the MAR index for the combined rural areas (0.028). However, MAR indices varied between sample sites and areas. It has been concluded that antibiotic resistant *E. coli* may offer an index of water quality as related to source.

E. coli isolates collected from environments considered to have low and high enteric disease potential for humans were screened against 12 antibiotics in order to determine the prevalence of multiple antibiotic resistance among the isolates of these environments. It is interesting that the MAR index for isolates from human raw sewage was greater than the index found among isolates recovered by direct anal swabbing. There may be several factors contributing to this observed differences, such as high concentration of nutrients and exchange of plasmid in the sewage system. This assumption is in agreement with observations made by Grabow et al. (68, 69) who demonstrated that plasmid exchange readily occurs between *E. coli* and other coliform bacteria in the stagnant areas of wastewater systems. He also observed that the MAR index of *E. coli* isolates from wild animals was generally low, while human and poultry isolates had higher MAR index (93).

Al-Ghazali et al. (3) performed a study in six sites along the Al-Khair river in Baghdad for monitoring water pollution. *E. coli* was used as a pollution indicator, and was tested for antibiotic resistance, multiplicity of resistance, and for transfer of antibiotic resistance determinants. For this study, a total of 500 *E. coli* were isolated, identified and tested for resistance to 12 antibiotics. Most of the isolates (92%) were resistant to ampicillin. In addition, a high proportion of antibiotic-resistance was observed for tetracycline, streptomycin, kanamycin and gentamicin. Investigation of MAR among *E. coli* isolates revealed that double resistance was dominant. In addition, ampicillin, carbenicillin and ampicillin gentamicin were predominant MAR patterns. They also divided antibiotic-resistant *E. coli* into two groups on the basis of degree of MAR, where group I was a high proportion (80%) of strains, including those that were resistant to 1 3 antibiotics, whereas group II included strains resistant to 4-7 antibiotics.

In another study, *E. coli* strains were isolated from Sauce Chico River waters and Bahia Blanca estuary waters and sediments in Argentina. Resistance to seven antimicrobial drugs was assessed. Resistance was most common for streptomycin, sulphamide and tetracycline. Isolates from fresh water showed lower resistance than those from estuarine areas receiving domestic sewage, except for sulphamide. This may indicate that there was a greater contribution from HS through sewage rather than animal sources of drug resistant *E. coli* strains (17). A similar study was conducted in Northern India to assess the spectrum of drug resistance prevalent in *E. coli* isolates from human and animal sources. In this study, *E. coli* isolates were tested for susceptibility to 9 antimicrobial drugs Animal isolates were less resistant to drugs than those from man (134)

The bacteriological quality of selected rural water supplies in Port Harcourt in Nigeria was monitored over a 3 month period by Antai (13) He reported that the most frequently isolated coliforms were *E coli Enterobacter aerogenes* and *Klebsiella pneumoniae* Coliform contamination was greater in well water than in river or stream samples Antibiotic sensitivity test revealed that 17 5 27 2% of *E coli* strains were resistant to three or more antibiotics *E coli* isolated from well water samples exhibited the greatest degree of multiple resistance Some strains were resistant to all six antibiotics However there was no correlation between resistance and water source

Niemi et al (110) also reported the distribution of *E coli* resistance to ampicillin, chloramphenicol sulfonamides, tetracycline and streptomycin among fecal coliforms in sewage, surface and sea water The incidence of resistant strains varied significantly in the water samples It was high in untreated domestic sewage and in some brackish and lake waters Bacterial resistance to different antibiotics varied from one water sample to another Again, no associations were observed between resistance and water source The species composition varied considerably in different water samples, and a correlation was observed between the relative frequency of *Klebsiella* species and incidence of ampicillin resistance

In another study, the MAR index of *E. coli*, isolated from water and sediments in the Bhavani river in India was used to assess the quality of the river, and the risk of contamination. It was found that 67% of *E. coli* were resistant to one or more antibiotic tested. Resistance to penicillin G, novobiocin, bacitracin and neomycin was most dominant. The incidence of MAR *E. coli* was most predominant in the water and the sediments, the MAR index was between 0.471 and 0.642. It was concluded that the river was contaminated with fecal bacteria originating from sources such as night soil, and commercial poultry farms. Based on this study the quality of river water was not safe for drinking and required treatment before use [62]. Hatha et al. [75] also isolated *E. coli* from water, sediment and fish of the Bhavani River, tested for antibiotic resistance for 10 antibiotics and found that 62% isolates were resistant to more than four antibiotics. Levels of resistance to bacitracin, penicillin G, and novobiocin were generally high, whereas those to polymyxin B and chloramphenicol were much lower. Multiple antibiotic resistant *E. coli* were noted in all samples, 95% of the MAR strains originated either from man or cattle. Al-Jebouri [4] isolated *E. coli* over a month period during 1984 from healthy human adults, raw sewage and the sewage-polluted Tigris river in Mosul, Nineva, and analyzed for MAR. More than 40% of the isolates were antibiotic resistant and of these 77.1% were resistant to more than one antibiotics. Results were similar for healthy individuals and those from raw sewage, lower resistance was found among *E coli* strains from polluted river water

Fatty Acid Methyl Ester Profiles (FAME)

To solve the problem of bacterial identification encountered in food pharmaceutical industries and many other laboratories, simple and rapid methods applicable to all bacteria have been developed Among these methods gas chromatographic analysis of cellular fatty acids is a simple characterization method Commercial identification systems based on this analysis are now available (Hewlett Packard Microbial Identification system (MIS), Avondale PA MIDI Microbial Identification System, Newark, DE) With controlled culture conditions, FAME profiles obtained are stable and produce "fingerprints" of bacteria This has led to the use of FAME profiles as a key method for bacterial taxonomy Advantages are that fatty acid metabolism is constitutive and directed by the chromosome. It has also been found that fatty acid profiles are not prone to alteration by moderate genetic mutations, and closely related bacteria have very similar fatty acid profiles Thus cluster analysis of cellular fatty acid profiles can be used to effectively group bacteria Analysis of cellular fatty acids tests actual cellular materials, in contrast to traditional biochemical methods that measure cellular density or color changes due to pH (59)

It has been observed that factors such as temperature, pH, salt content of the medium, and growth stage at the time of harvest effect FAME profile (60) Linder and Oliver (95) reported that colonies for extraction of fatty acid should be harvested in the late log phase when stable fatty acids are produced. However, cells should not be harvested too late in the log phase. For example, in *E. coli* as the cells aged the percentage of saturated fatty acids increased from 49 to 89.5%, the unsaturated fatty acids decreased from 45 to 17%, and previously undetected long chain fatty acids appeared. A similar trend was also observed for other bacteria. Yet, fatty acid composition can be consistent if cultural conditions are strictly standardized.

Cellular fatty acids are usually defined as those that are 9 to 20 carbons in length, including the majority of fatty acids in the cell membrane in the form of glycolipid and phospholipid. Most bacteria synthesize fatty acids in a chain length of 10 to 19 carbon units with predominant chain lengths of 16 to 18. The acids are named according to the number of carbons in the chain, the type of functional group, and double bond location (131).

In gram-negative bacteria, in addition to the cell membrane fatty acids, the lipid A component of the lipopolysaccharide (LPS) is a major source of fatty acids. In gram-positive bacteria, it is lipoteichoic acids. The type of fatty acid produced by a particular organism is indicative of specific biosynthetic pathway used to make that fatty acid. More than one hundred fatty acids have been identified, and the amount and the type of fatty acid present in the cell is unique to an organism (106).

In any organism, 8 to 15 fatty acids are usually predominate. The most common are saturated, straight chain or monounsaturated acids with a single double bond. Gram-negative bacteria usually contain a higher percentage of saturated and monounsaturated fatty acids with even number chain lengths whereas gram-positive bacteria have more branched chains, odd numbered chain lengths, and lower levels of straight chain saturated fatty acids (106).

Although MIS was designed for microbial species identification, it has been suggested that the system may also be used for epidemiological typing of strains (31, 107). Mukwaya and Welch (107) used MIS to track the sources of *Pseudomonas cepacia* strains isolated from cystic fibrosis treatment centers, and Birnbaum et al. (31) used the MIS to estimate the relatedness of a large number of coagulase-negative *Staphylococcus* strains. Recently, Steele et al. (143) analyzed whole cell fatty acid profiles of verotoxigenic *E. coli* and *Salmonella enteritidis* by gas liquid chromatography and observed sub groups within each species. They also observed variability in fatty acids profiles with repeated preparations of the same strains, thus limiting the usefulness of this system for sub-grouping.

Wauthoz et al. (155) analyzed fatty acid composition and intraspecies similarity of food borne bacteria, mostly *Bacillus, Enterobacteriaceae*, lactic acid bacteria, and *Staphylococcus*. They observed low coefficient values within each of the four groups of strains investigated, which stressed the importance of analyzing a high number of reference strains in each species. Intraspecies similarity was best under optimal growth conditions. Finally, interspecies similarity was compared to intraspecies similarity. The best results of discrimination were obtained for the heterogeneous genus *Bacillus*, for which the 12 species studied could be separated into 6 clusters at a similarity level of 79. However, only 3 clusters were formed for the 10 *Enterobacteriaceae* species studied. In contrast, the genus *Salmonella*, coagulase-positive and negative *Staphylococcus* strains could not be differentiated. They suggested that since the fatty acid composition did not usually appear specific to a single species, characterization of food borne bacteria by the analysis of their cellular fatty acids should be used with other taxonomic methods. However, these results are in contrast to those of other studies where MIS was used to examine the relatedness of coagulase-negative *Staphylococcus* (31) and to differentiate the *Aeromonas* strains from *Vibrio* strains (151). These authors found the system to be a useful screening tool compared to other more time consuming and expensive typing methods.

Serogrouping

The most widely used and perhaps best known method of identifying individual strains of *E. coli* is based on an immunological reaction. Three surface structures corresponding to the O, K, H, antigens form the basis of the identification. From literature, it has been found that K antigens are only present in capsulated *E. coli* and have served a minor role in serotyping. Therefore, serotyping has been mainly based on O and H antigens (protein of flagella). H antigen is only present in actively motile *E. coli*. O-antigen is a thermostable surface structure found in all smooth *Enterobacteriaceae*. It constitutes O-specific polysaccharide of cell wall LPS and is determined by chromosomal genes (118).

The first successful attempt to classify *E. coli* by serological methods was carried out by Kauffmann (87, 88) who was able to subdivide *E. coli* strains into 20 O groups. He used a boiled culture for O antiserum production and agglutination tests. It was soon extended by Knipschildt (89) to contain 25 O-groups. Since then, many O antigens have been added, increasing the present antigenic scheme to 181.

Little is known about the distribution of different serogroups. The best examined are strains from humans and common domestic animals in temperate climates. It is not yet possible to state whether there are significant differences in O-group prevalence between different animal groups. However, some investigators reported differences in O groups between humans and animals (74, 115).

Although the distribution of different *E. coli* strains identified by serotype seems to be different in humans and animals, many serotypes found in normal humans can also be found in other organisms (118). Some authors have indicated that differences in O serogroup distribution exist, e. g. between Europe and the United States (61), even within different areas in London (71). Others have suggested that prevalence rates of certain O-groups could vary with time in the same area (100).

Even though a very large number of serogroups exist in nature, only a few *E. coli* are associated with most disease It is known that some antigens and some O K H serotypes are more common than others It is also known that strains causing extra intestinal disease originate from normal intestinal flora (97) in contrast to the serotypes from extra intestinal disease the diarrheal serotypes are generally not found in normal gut (117) Few studies have been carried out on *E coli* isolated from UTI in dogs and cats It has been found that a limited numbers of antigens and serotypes are involved with some of them found in man such as O6 H1, O4 H5 O6 K53 H1, and O6 K13 H1 (55, 157)

Bettelheim et al (23) compared serotype distribution of *E coli* isolated from animal feces, human feces and meat and found that there are marked differences in serotypes distribution in strains from man and animals The meat strains generally resembled animal strains These results suggest either, animal strains of *E coli* are not reaching the general human populations to any great extent, or if they do so fail to colonize the bowel

Ribotype (RT)

Ribotyping, relatively a recent development in DNA analysis, is the study of the distribution of the restriction sites throughout the rRNA genes It has been successfully used for characterization of microorganisms belonging to different genera (9, 16, 70, 121, 125 145) However, minimal research has been done on differentiating *E coli* from sources by RT

Picard et al. [124] conducted a study to compare carboxylesterase type B_1 (less virulent) and B_2 (highly virulent) strains of *E. coli* isolated from extra intestinal infections by this technique. They digested genomic DNA with *Hind* III, *Eco*RI, *Bam*HI, *Bgl*II, and detected digested DNA with ^{32}P labeled 16S and 23S rRNA probes. It has been found that B_2 strains appear to be less heterogenous than B_1 strains (8, 4, 1, and 3 RT patterns for *Hind*III, *Eco*RI, *Bam*HI, and *Bgl*II, respectively, for B_2 strains, whereas 13, 10, 7, and 9 respectively for B_1 strains). The combination of patterns resulting from the four digestions revealed 23 distinct RTs among the 28 strains. Nine RTs were detected in B_2 strains, and 14 in B_1. The B_2 strains were also separated from B_1 by possession of 5.5 kb, 9.5 kb, 22.5 kb fragments and 3.0 kb, 3.4 kb, 2.0 kb fragments, respectively, when digested the DNA by *Hind*III and *Eco*RI. Consequently, these results demonstrated that virulent *E. coli* strains type B_2 are genetically distinct from B_1.

A similar study was carried out to differentiate *Fusobacterium necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme* isolated from bovine ruminal contents and liver abscesses by RT. In this case, genomic DNA was digested with *Eco*RI, and showed that two distinct fragments of 2.8 and 4.3 kb differentiated the two subspecies. Regardless of origin, only *F. necrophorum* subsp. *necrophorum*, a virulent subspecies, had a 2.3 kb fragment whereas *F. necrophorum* subsp. *funduliforme*, a less virulent subspecies, had a 4.3 kb fragment (112). Ribotype was also used to separate

E. coli strains isolated from different extra intestinal infections, stool of healthy persons, 20 different RTs were detected. No correlation was described for RT and source. However, these authors observed that RT was able to differentiate different serogroups of *E. coli* (14). Conversely, other investigators reported that RT was not a suitable method to differentiate different serogroups of *E. coli* (102, 155).

Alos et al. (6) used RT to trace nosocomial transmission of *E. coli* K1 in a neonatal unit. They isolated *E. coli* K1 from premature neonates, a ventilation system, and from a storage shelf of the ward from December 1991 to June 1992. Eight *E. coli* K1 strains isolated from infants in the nursery between 1989 and September 1991 were included as controls. It was found that the majority of the strains from premature neonates colonized with *E. coli* K1 were RT I, as were isolates from the ventilation system and storage shelf. An interesting finding was that isolates from feces and milk of three mothers were also RT I. Eight control isolates belonged to seven different ribotypes, but not RT I. It was concluded that *E. coli* K1 was transferred from mother to the newborn infant. Moreover, nosocomial transmission has also been suggested.

Paros et al. (121) performed a significant research to compare human and bovine *E. coli* O157:H7 by bacteriophage λ RFLP profiles. In this study, they isolated *E. coli* from human patients and bovine reservoirs, digested the DNA with *Eco*RI and identified digested DNA with a bacteriophage-λ probe. Among the 72 *E. coli* O157:H7 human and bovine strains, 23 λ RFLP were examined.

Eleven patterns accounted for 81% of the isolates. They found that 90% of the human *E. coli* O157:H7 did not match bovine strains.

Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is a genotypic method that directly detects variations in nucleotide sequences in the chromosomal DNA. It has been shown to resolve genomic restriction fragments ranging from microorganisms responsible for nosocomial infections (5), *Vibrio* species colonizing oysters (34, 147), *Listeria* species in vegetables (42), to coliforms isolated from water distribution systems and source water (46). This technique utilizes the discriminatory power of restriction enzymes and separates large DNA fragments ranging from 50 to over one million base pairs. From the literature it is apparent that PFGE has been mainly used in epidemiological studies especially for *E. coli* O157:H7 (19, 82). However, few studies have determined association between *E. coli* PFGE profiles and source.

Arbeit et al. (14) investigated if PFGE could differentiate isolates within similar evolutionary lineages. For this study, isolates were obtained from bacteremic patients identified over 32 months. For each patient, they obtained primary source isolates (urinary or biliary tract), concurrent blood isolates, and then analyzed these by PFGE using *NotI*. They found that isolates from different patients exhibited distinctive restriction fragment length polymorphisms (RFLP). In contrast, isolates from different sites with the same patient had identical restriction profiles. These results indicated that PFGE can resolve recent evolutionary divergence within *E. coli* lineages and, consequently, offers a powerful means of molecular epidemiology. Gordillo et al. (64) also reported that PFGE is a powerful tool to differentiate different serogroups of *E. coli*. They observed that PFGE profiles of serogroup O143 were different from those of non-O143 serogroups.

Herbein et al. (77) used PFGE to investigate nonpoint fecal coliform sources in tidal inlets on the Eastern shore of the Chesapeake Bay. They characterized *E. coli* strains isolated from wildlife, humans, tidal waters, using *Not*I restriction endonuclease. It was observed that PFGE profiles varied from animal to animal, and that PFGE profiles of resident wildlife showed high homology with *E. coli* in tidal waters. PFGE profiles of HS isolates were different from strains isolated from resident wildlife, and they concluded that PFGE profiles may be an adjunct to field ecological data in identifying nonpoint source pollution. In contrast, other investigators did not find any correlation between coliform bacteria and their source using PFGE (46).

Plasmid Profiles

Antibiotic resistant bacteria are world wide problem. Various authors have reported high degrees of antibiotic resistance in bacteria from a variety of sources (21, 120, 133). Numerous studies of plasmids coding for antibiotic resistance in enteric gram-negative bacteria have been reported. Plasmids are responsible for multiple drug resistance in *E. coli*. Self transmissible heavy plasmids of molecular weights more than 30 MDa are associated with this resistance (133). Heavy plasmids have also been reported to be responsible for multiple resistance in *Salmonella* and *Shigella* isolates (84). Plasmids of 40-80 MDa have also been associated with virulence in *E. coli* (45).

Baya et al. (20) examined the association of plasmids and antimicrobial resistance to marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples and found no significance correlation between number of plasmid bands, or average plasmid molecular weight, and antibiotic resistance pattern. Out of 18 strains, 10 had no detectable plasmids and no specific size range of plasmid DNA correlated with any resistance combination. This work was supported by other investigators (51, 120). However, Murdoch et al. (108) observed a correlation between the presence of large plasmid (150 kb or larger) and ampicillin resistance in clinical *E. coli* isolates. Levy et al. (98) also conducted a study to determine whether MAR was associated with plasmid profile. For this study, they isolated *E. coli* and other members of *Enterobacteriaceae* from diarrheal specimens in hospitalized children in Indonesia, and found that all strains were multiple resistant, many of them were resistant to six or more antibiotics. Most resistance could be transferred to laboratory *E. coli* strains and were carried on large plasmids. They also observed that resistance was carried on more than one plasmid. The presence of multiple resistance on a single or multiple plasmids in the same organism has been linked to prolonged use of a single antibiotic (95). The results suggest that long term use of antibiotics leads to emergence of multiple resistant *Enterobacteriaceae*

Similarly Singh et al (135) studied 109 highly resistant strains of *E coli* representing various human and animal diseases for transfer and non-transfer of resistance It was found that 47.7% of the isolates transferred drug resistance partially or completely, by conjugation whereas 32% transferred resistance by mobilization. A majority of transconjugants were multiple resistant Transfer was common for ampicillin resistant determinants while minimum for trimethoprim sulfathiazole and observed more frequently in man (64%) as compared to animal strains (33.8%). By chemical treatment, non-conjugable and non mobilizable resistant strains were cured of resistance, thereby indicating the involvement of plasmids They concluded that exchange of plasmid-mediated resistance in human and animal sources of *E coli* was not uncommon.

MATERIALS AND METHODS

Experimental Design

The experimental protocols were segregated into three main phases I Collection of surface water, sewage effluents, human and animal feces. II Analysis of *E coli* isolates for MAR, FAME, O-serogroup, RT, PFGE, and plasmid profiles; and III Correlation of these characteristics with the source of the isolates.

Sampling Sites and Collection

Estuarine surface water (500-750 ml) was collected on an out going tide at monthly intervals over one year, in duplicate, in sterile Whirl pack bags (Fisher Scientific, Atlanta, GA). Specifically, ANERR surface water was collected from three sites in the northeast region of East Bay which receives extensive drainage from protected marshlands, but no known HS pollution (Fig. 1 and 2). Samples (250-500 ml) of sewage treatment plant effluents were collected from municipal treatment plants on the east and west shores of Apalachicola Bay (Fig 1 and 2). *E coli* isolates were also collected directly from feces of human volunteers living in Apalachicola, FL and from animal feces deposited within the ANERR wildlife reserve.

Figure 1 Major features of the Apalachicola National Estuarine Research Reserve (ANERR)

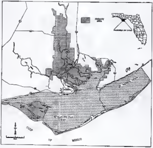
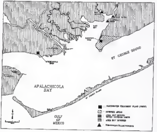

APALACHICOLA
BAY
GULF
OF
MEXICO

Figure 2. Major features and sample sites of the Apalachicola National Estuarine Research Reserve (ANERR)

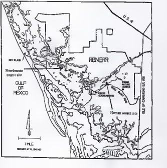

ST GEORGE SOUND
EAST BAY
APALACHICOLA WWTP
APALACHICOLA BAY
ST GEORGE ISLAND
GULF OF MEXICO
WASTEWATER TREATMENT PLANT (WWTP)
SEWERED AREAS
AREA NOT SEWERED

Surface waters impacted by human and wildlife were also collected from RBNERR. In this case, HS surface water was collected from Henderson creek (receives septic tank seepage) which bisects the reserve and is also moderately affected by wildlife, especially manatees during the winter season. Nonhuman source surface water was collected from the Blackwater area, which receives drainage from nearby land that is influenced by wildlife populations. The map of Rookery Bay (Fig. 3) is marked to show the collection sites. After collection all samples were stored at 4°C and transported via overnight courier.

Isolation and Identification of *E. coli*

Sample preparation and bacteriological tests for isolation of *E. coli* were performed using established procedures (7, 8, 88). All samples were processed within 24 h of collection. A predetermined water volume, based on an initial measurement of the *E. coli* MPN, was filtered through a 0.2 μm filter (Gelman Sciences, Ann Arbor, MI). Filters were placed on MacConkey agar (Difco Laboratories, Detroit, MI), incubated at 35°C for 18 h, then all lactose fermenting *E. coli*-like colonies screened using 4-methylumbelliferyl-β-D glucuronide [(MUG), Sigma Chemical Co, St. Louis, MO] (78). Sewage and feces samples were diluted in 10-fold serial increments. 0.1 ml of each dilution plated on MacConkey agar, and incubated at 35°C for 18 h. Typical *E. coli*-like colonies were screened using MUG. Presumptive *E. coli* isolates were confirmed by standard biochemical tests (7, 8).

Figure 3: Major features and sample sites of the Rookery Bay National Estuarine Research Reserve (RBNERR)


U.S. 4
KEY ISLAND
RBNERR
Non-human source site
GULF OF MEXICO
ROOKERY BAY
BRIGGS NATURE CENTER
HEADQUARTERS
SHELL ISLAND ROAD
Human source site
ISLE OF CAPRI ROAD S.R. 951
1 MILE

Maintenance of Culture

All culture were maintained at -70°C in 12% glycerol TSB medium, composed of 12% glycerol in tryptic soy broth (TSB, Difco). Prior to experimentation bacteria were transferred from 12% glycerol TSB to tryptic soy agar (Difco), and incubated at 37°C.

Multiple Antibiotic Resistance (MAR)

MAR indices were performed by the method of Kaspar et al. (88) using selected antibiotics typically associated with animal feed and/or clinical treatments. Briefly, stock solutions of antibiotics were filter sterilized and stored at 5°C in the dark. The following final concentrations were used: 10 µg/ml ampicillin, 25 µg/ml chlortetracycline, 25 µg/ml kanamycin, 25 µg/ml nalidixic acid, 50 µg/ml neomycin, 25 µg/ml oxytetracycline, 75 U/ml penicillin G, 12.5 µg/ml streptomycin, 500 µg/ml sulfathiazole, and 25 µg/ml tetracycline. Aliquots of stock solutions were added to tempered (46°C) Mueller Hinton agar (Difco), mixed, poured into petri dishes and stored at 5°C for no longer than two weeks. *E. coli* isolates were grown in 96 well plates containing tryptic soy broth (Difco) at 35°C for four to six hours, replica plated onto antibiotic containing agar and control plates without antibiotic, and incubated at 35°C for 18 h. *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were used as positive (resistant to all antibiotics tested except for sulfathiazole, *E. coli* ATCC 25922 or *Klebsiella pneumoniae* ATCC 13883 was used as positive control for sulfathiazole) and negative (sensitive to all antibiotics tested) controls, respectively. Isolates were recorded as resistant to an antibiotic if growth, measured with a ruler, was indistinguishable from that on the control plate without antibiotic; more than 10 to 15% reduced growth was recorded as sensitive reaction to the antibiotic, although growth was normally reduced greater than 90%. The MAR index (number of antibiotics the isolate was resistant to ÷ total number of antibiotics tested) was determined for each isolate.

Fatty Acid Methyl Ester (FAME) Extraction

Whole-cell fatty acids were extracted and analyzed as methyl ester derivatives by Mukwaya and Welch (107). In brief, bacteria were cultured on tryptic soy broth agar (TSBA; BBL, Cockeysville, MD) for 24 ± 2 h at 28°C. The collected bacteria were saponified with 1.0 ml of 1.2 N NaOH in 50% methanol at 100°C for 30 min. Saponified fatty acids were methylated by adding 2.0 ml of 6.0 N HCl in methanol and heating to 80°C for 10 min, followed by rapid cooling at room temperature. FAMEs were extracted with 1.25 ml hexane/methyl t-butyl ether (1:1 v/v), discarding the acidified lower phase. The extract was washed and neutralized with 3.0 ml of 0.3 N NaOH. Extracts were then transferred to a chromatography vial and capped. With each batch of extractions, a reagent blank was included along with a control strain of *Xanthomonas maltophilia* grown on tryptic soy broth agar for 24 h at 28°C. The profile of this bacterium is well characterized and was included to ensure reproducibility among extraction of different batches.

Gas Liquid Chromatography

Fatty acid methyl esters were separated using a Hewlett Packard 5890 gas liquid chromatographed fitted with a 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column, and a flame ionization detector, a model 3392A integrator, a model 7673A automatic sampler, and a model 310 computer. Initial temperature of the oven was 170°C which was increased at a rate of 5°C/min to a final temperature 270°C. The carrier gas was H_2 at a flow rate of 20 ml/min. The peaks were automatically integrated, and fatty acid identities and percentages were calculated by comparing their retention times to those of a bacterial fatty acid standard (bacterial acid methyl ester mix CP 4-7080 Supelco, Inc., Bellefonte, PA).

O-Serogrouping

The *E. coli* isolates were serogrouped for 181 specific O-antisera by standard methods at the *E. coli* Reference Center, Pennsylvania State University (159).

Preparation of Antigen

E. coli isolates were grown on Veal Infusion Yeast Extract agar (Veal Infusion, 25 g; Yeast Extract, 2 g; Dextrose, 1 g; Agar, 1 g; dH_2O, 1 L) and incubated at 37°C for 18 to 24 h. For each plate, a corresponding 13x 25 mm test tube was filled with 5 ml of 0.5% formal saline (NaCl, 8 g; 37% formaldehyde, 5 ml; dH_2O, 1 L); all growths were completely removed from the plate by a cotton swab, suspended in appropriate test tube, and heated at

100°C for 2 h After cooling, another tube containing 3 ml of 0 6% formal saline was placed in front of the antigen and 3-4 drops of heated culture added to approximate a MacFarland # 3 standard

Microtiter Screening Test

Twenty microliters of each antisera and 180 μl of the diluted antigen were added to each well of a V bottom tissue culture plate Then plates were placed in a humidity chamber and incubated at 50°C for 24 h and read with the aid of a microtiter plate viewer A negative reaction appeared as a white button and a positive reaction was cloudy to clear Confirmation and Identification were continued if the reaction was positive If there was no agglutination the antigen was reheated at 120°C for 60 min and the slide procedure repeated

Confirmation and Identification

Ten microliters of antisera was added to the appropriate well in row A of 96 well microtiter plate Then, 190 μl of phenol saline (NaCl, 6 g, phenol, 6 ml, dH_2O, 1 L) was added to row A and 100 μl of phenol saline added to rows B through H and mixed row A Serial dilutions were continued through row G, but not H. Then 100 μl of antigen were added to rows A through H, the plate was placed in a humidity chamber and incubated overnight at 50°C Reactions were graded as follows: a button in the bottom of the well was a negative reaction, and slight cloudiness with no button was a positive reaction No visible button through row D (1/640) indicated a positive result Row H was negative i.e a visible button, for the test to be valid

Antisera Preparation

All antisera were maintained in a 1 2 dilution of glycerol and refrigerated at 4°C It was used at a final dilution of 1 640 Antisera were diluted with phenol saline O-Microtiter confirmation antisera were used directly from stock bottles Well A had a final titer of 1 80 and well G a final titer of 1 5120 Well H was used as a negative control

Ribotype (RT)

Ribotype was determined for *E coli* as described by Schoonmaker et al (132) with some modification *E coli* ATCC 9637 was used as a control strain

Extraction of DNA

DNA was extracted by the method of Sambrook et al (130) Briefly, *E coli* were grown in Brain Heart Infusion Broth (Difco) late log phase cultures were pelleted at 3000 x g for 15 min and treated with lysozyme (10 mg/ml, Sigma) proteinase K (10 mg/ml, Fisher Biotech Fair Lawn, NJ) and RNase (150 µg/ml, Fisher) at 37°C for 30 min DNA was extracted with chloroform/isoamyl alcohol (24 1), precipitated with 100% isopropanol, washed once in 70% ethanol, recovered, dried and resuspended in TE buffer (0 1 M Tris, 0 1 M EDTA, pH 8 0)

Determination of Concentration of DNA

DNA concentration was determined using a TKO 100 Mini-Fluorometer according to the maunufacturer's instruction (Hoefer Scientific Instruments San

Francisco, CA). Two microliters of 100 μg/ml calf thymus DNA was used as a standard.

Restriction Enzyme Analysis

A known concentration (100 μg/ml) of DNA was digested with several restriction enzymes, e.g., *Hin*dIII, *Eco*RI, *Sal*I, *Bgl*I (Gibco BRL, Gaithersburg, MD) in accordance with the manufacturer's instruction. Digested DNA was separated in 1.0% agarose gel at 30 V for 16 h in TBE buffer (0.9 M Tris-borate, 0.002 M EDTA), stained with ethidium bromide (5 μg/ml) and photographed under short-wave UV light. A *Hin*dIII digest of bacteriophage lambda (Promega Corp., Madison, WI) was used as a molecular weight marker.

Southern Blot Analysis

After electrophoresis of restriction digested DNA, agarose gels were denatured in 0.5 M NaOH, 1.5 M NaCl for 35 min and neutralized in 0.5 M Tris-HCl, pH 7.0, 1.5 M NaCl, 0.001 M disodium EDTA for 45 min. DNA was then blotted from the gel to nylon membranes (Bio Rad Laboratories, Hercules, CA) using a vacuum blotting system (VacuGene™ XL, Pharmacia Biotech, Piscataway, NJ), and fixed with short-wave UV light for 5 min (141).

Preparation of Probe

E. coli 16S and 23S rRNA were reverse transcribed into cDNA with avian reverse transcriptase (Boehringer Mannheim Corp., Indianapolis, IN), then labeled with digoxigenin-dUTP according to the manufacturer's instructions (Boehringer Mannheim Corp.).

Hybridization and Detection

Membranes were prehybridized at 42°C for two h then hybridized with dig labeled probe using 25 ng DNA per 15 x 15 cm^2 filter at 65 °C for 16 h. After hybridization membranes were washed twice for 5 min each with 2 x SSC (sodium chloride, sodium citrate) 0 1% SDS (sodium dodecyl sulfate) at room temperature and twice for 15 min each with 0 5 x SSC-0 1% SDS at 65°C, reacted with antibody and visualized using nitroblue tetrazolium and 5-bromo-4-chloro 3-indolyl-phosphate for colorimetric detection following the manufacturer s instructions (Boehringer Mannheim Corp)

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE analysis was performed as described by the method of Buchrieser et al (34) A single colony of each isolate was inoculated into 10 ml tryptic soy broth (Difco), and incubated overnight at 37°C Cells were harvested washed and suspended in agarose plugs as previously described (33) To lyse cells and degrade cellular proteins agarose plugs were incubated at 50°C overnight in proteinase K solution (Fisher Biotech 2 mg/ml in 0 5 M EDTA and 0 5 % N-Lauryl sarcosine, Sigma) Plugs were then treated with phenylmethylsulfonyl fluoride (Sigma) and washed three times in TE buffer The genomic DNA embedded within agarose plugs was digested with *Sfi*I and *Not*I restriction endonucleases as recommended by the manufacturers (New England Biolabs Beverly MA, Stratagene, La Jolla , CA, or Promega Corp) High molecular-weight restriction fragments were resolved with a CHEF DR II pulsed field system (Bio Rad Laboratories, Richmond, CA) using 1.0% pulsed-field certified agarose gel (Bio-Rad). An electrophoresis regimen of 200 V for 24 h at a temperature of 18°C and a switching time from 1 to 40 sec was used to separate fragments. Low range PFG and lamda ladder PFG (New England Biolabs) markers were used as standard. Gels were stained in ethidium bromide solution (Sigma, 5 μg/ml) and photographed under short wave UV light. The size of the DNA fragments was compared visually.

Isolation of Plasmid DNA

Plasmids were prepared by the alkaline lysis method described by Birnboim and Doly (32). In brief, a single bacterial colony was inoculated into 5 ml of Luria broth (LB) and inoculated overnight at 37°C. Approximately, 1 ml of each overnight broth culture was transferred into 1.5 ml eppendorf tube and centrifuged for one min at 10,000 x g/min at room temperature. The supernatant were discarded and pellets were suspended in 100 μl of 2 mg/ml lysozyme, 10 mM EDTA, 25mM Tris, 5mM glucose, pH 8.0. After 30 min incubation at room temperature, 200 μl of solution containing 0.2N NaOH 1% SDS was added and the tube vortexed gently. The tube was maintained for 5 min at room temperature and then 150 μl of 3 M sodium acetate, pH 4.8 was added. The contents of the tube were gently mixed by inversion for a few seconds and during that time a clot of DNA formed. The tube was maintained on ice for 15 min and centrifuged at 10,000 x g for 5 min, and 400 μl of supernatant were transferred to a second eppendorf tube. One ml cold ethanol was added and the tube held on ice for 15 min. The precipitate was collected by centrifuging for 15 min and decanting the supernatant. Pellets were rinsed with 150 µl 95% ethanol. After drying at room temperature for 10 min, pellets were suspended in 40 µl of TE buffer and 1 µl of 10 mg/ml RNase added and mixed by inversion.

Agarose Gel Electrophoresis of Plasmid DNA

Agarose gel electrophoresis and molecular size estimation were performed as described by Meyers et al. (105). Briefly, 3 µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanole, 40% sucrose) were added to 15 µl of plasmid DNA and applied to a 0.8% horizontal agarose gel and electrophoresed at room temperature at 120 V for 3 h in TBE buffer. After electrophoresis, gels were stained with ethidium bromide (5 µg/ml), visualized, and photographed under short wave UV light. Approximate plasmid molecular weights were determined by comparing their relative migration rates with those of plasmids of known sizes: pTr 99c (3.6 kb), pUC13 (2.7 kb), pUC 19 (2.7 kb), pBR 322 (4.4 kb), *Salmonella typhimurium* UF 021 (90 kb), and pYA 438 (52 kb), which were kindly provided by Dr. P. Gulig, University of Florida.

Statistical Analysis

Significant differences between antibiotic resistance patterns of HS and NHS isolates were determined by a two-sided test of binomial proportion ($P < 0.05$). Data were converted to binary code and interisolate relationships measured by the Euclidian metric, average linkage method (139). Relationships were examined using cluster analysis and demonstrated using plots of principal-components similarity coefficients. Computations were performed using SAS™/Base and SAS™/ETS software (63, 142).

The quantitative data obtained from the FAME profiles were used as the basis for numerical analysis. Peak area values for each fatty acid were calculated as percentages of the total peak area to eliminate the effect of inoculum size variation. Differences in the amount of the fatty acids between HS and NHS isolates were evaluated statistically by using the two-sided test of binomial proportion ($P < 0.05$). Similarities were calculated with the generalized similarity coefficient of Gower (66) and the coefficient based on the Euclidian distance between the pairs of bacteria. Clustering of strains was achieved by the unweighted pair group method for arithmetic averages (138) with a program provided in the HP Library Generation Software (Hewlett-Packard Co., Avondale, PA), resulting in a dendrogram. Principal component analysis of the quantitative fatty acid data was performed with the above mentioned statistical program, and the results were plotted graphically in two dimensions.

The significance differences between the predominant O serogroups of HS and NHS isolates were also determined by a two sided test of binomial proportion ($P < 0.001$).

RT profiles were measured using DNA proscan™ software (Nashville, TN). DNA fragments for each strain were translated into binary code. Similarity indices were determined using Dice's coincidence index (43), where restriction endonuclease digestion profile similarities (S_{xy}) for individuals x and y equals the numbers of common fragments in their DNA profiles (n_{xy}) divided by the average numbers of fragments exhibited by both individuals $(S_{xy} = 2n_{xy})n_x + n_y)$ Relationships among isolates were examined using cluster analysis Data management and analysis were performed in the SAS™ (SAS Institute, Cary, NC) system and cluster dendrograms were plotted using S plus (Statistical Services, Inc., Seattle WA) software

RESULTS AND DISCUSSION

Multiple Antibiotic Resistance (MAR)

A total of 765 *E. coli* were isolated and identified from ANERR, and analyzed for MAR. It was found that 82% of the isolates were resistant to one or more antibiotics (Tables 1 and 2). The percentage of isolates resistant to single antibiotics was significantly ($P<0.05$) higher for HS (94.8%) compared to NHS isolates (67.6%, Table 1).

Examples of single antibiotics which differentiated HS and NHS isolates at $P<0.05$ were ampicillin, chlortetracycline, kanamycin, nalidixic acid, neomycin, oxytetracycline, streptomycin, sulfathiazole, and tetracycline (Table 1). In contrast, there was no significant difference in resistance to penicillin G for HS and NHS isolates ($P=0.56$, Table 1).

The predominant single and MAR patterns of *E. coli* are shown in Table 2. In general, HS isolates showed higher resistance to single and combinations of antibiotics than NHS isolates. Specifically, 68 resistance patterns were observed for HS isolates compared to only 32 patterns for NHS isolates. Among HS isolates, relatively higher resistance was observed for chlortetracycline-sulfathiazole (33.7%) and chlortetracycline penicillin G

Table 1 Percentage of *E. coli* human source (HS) and nonhuman source (NHS) isolates resistant to antibiotics (ANERR)

Antibiotics	Percent of resistant strain		*P*-Value[a]
	HS (n=407)	NHS (n=358)	
Ampicillin	12.8	4.6	<0.05
Chlortetracycline	88.5	61.2	<0.05
Kanamycin	6.9	0.6	<0.05
Nalidixic acid	2.3	0	<0.05
Neomycin	3.2	0.3	<0.05
Oxytetracycline	10.3	3.4	<0.05
Penicillin G	38.6	37.7	0.86
Streptomycin	10.8	2.8	<0.05
Sulfathiazole	86.3	21.0	<0.05
Tetracycline	10.3	1.7	<0.05
Total	94.6	87.6	<0.05

[a] The *P*-value for HS and NHS isolates was determined by a two sided binomial test

Table 2. Predominant antibiotic resistance patterns of *E. coli* isolated from human source (HS), nonhuman source (NHS), human*, and animal feces

Antibiotics[a]	Percent with each resistant pattern			
	HS[b]	NHS[c]	Human[d]*	Animal[e]
C Su	33.7	16.4	40.0	ND
C P Su	14.6	1.7	ND	ND
C	7.9	6.4	16.7	ND
C P	7.4	28.2	ND	6.9
Su	2.2	1.4	6.7	ND
Other resistant patterns[f]	28.9	12.5	23.3	20.7
Sensitive to all antibiotics	5.4	32.4	13.3	72.4

[a] C: Chlortetracycline, P: Penicillin G, Su: Sulfathiazole
[b] n = 407
[c] n = 356
[d] n = 30
[e] n = 29
[f] Each of the other 59 patterns for HS, 28 patterns for NHS, 6 patterns for human*, and 3 patterns for animal feces isolates contained insignificant number of isolates are not shown
* Isolates obtained directly from human feces

Table 3. Percentage of *E. coli* isolates at different MAR indices

MAR index	HS (%)	NHS (%)
≤0.1	15.0	46.0
0.2	47.0	47.4
≥0.3	38.0	7.6

sulfathiazole (14.5%). In contrast, 28.2% of NHS isolates were resistant to chlortetracycline-penicillin G compared to only 7.4% of HS isolates.

Fifteen and 38% of HS isolates had MAR indices 0.1 or less and 0.3 or more, respectively. Conversely, 45 and 7.6% NHS isolates had MAR indices 0.1 or less and 0.3 or more (Table 3). In addition, the average MAR index for HS isolates was 0.25 compared to 0.13 for NHS. These findings coincide with Kaspar et al. (88) who found that urban areas had a greater range and frequency in MAR indices than rural areas. However, equal number of isolates from both sources had MAR index of 0.2 (Table 3). Therefore, samples in this range require careful scrutiny.

The relationship of antibiotic resistance pattern to HS isolates versus NHS, based on coefficients of similarity measured by Euclidian distance, is shown in Fig. 4 and 5. At a Euclidian distance of approximately 1.8, seven clusters were formed among HS isolates, designated as P_1 to P_7 (Fig. 4). In contrast, four clusters were observed for NHS isolates, designated N_1 to N_4 (Fig. 5). A covariance matrix of principal-components clearly showed more diversity among HS isolates (Fig. 6). In contrast, NHS isolates formed six focal groups (A-F). Clusters N_2 and N_4 were only found in groups A, B, and D. N_1 only in C, and N_3 only in E and F. These results indicate that the antibiotic resistance profile of *E. coli* is associated with its source of pollution in the ANERR. The unique foci of NHS isolates observed by covariance matrix (Fig. 6) also shows

Figure 4 Dendrogram of antibiotic resistance profiles for human source (HS) *E. coli* isolates determined by Euclidian metric, average linkage analysis. Clusters were defined at a distance of 1.8. Numbers on dendrogram represent antibiotic resistant pattern, not strain number. Row designations at bottom indicate the number of isolates in each cluster and the Euclidian distance is indicated left the plot

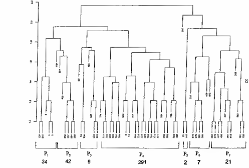

2.2
2.0
1.8
1.6
1.4
1.2
1.0
P_1
34
P_2
42
P_3
9
P_4
291
P_5
2
P_6
7
P_7
21

Figure 5 Dendrogram of antibiotic resistance profiles for nonhuman source (NHS) *E coli* isolates determined by Euclidian metric average linkage analysis Clusters were defined at a distance of 1 8 Numbers on dendrogram represent antibiotic resistant pattern not strain number Row designations at the bottom indicate the number of isolates in each cluster and the Euclidean distance is indicated left the plot

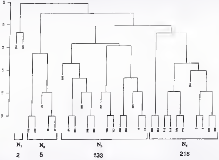
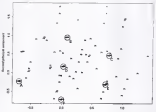

2.0
1.8
1.6
1.4
1.2
1.0
N$_1$
2
N$_2$
5
N$_3$
133
N$_4$
218

Figure 6 Two dimensional plot of antibiotic resistance patterns by principal-component analysis. Circles represent groups A to F. The specific cluster and associated number of isolates are: A, $N_4=117$, $N_2=3$; B, $N_4=59$, $N_2=1$; C, $N_1=2$, $N_3=7$; D, $N_4=36$, $N_2=1$; E, $N_3=13$; F, $N_3=13$


Second principal component
First principal component
-0.5
0.0
0.5
1.0
1.5
A
B
C
D
E
F

that NHS of *E. coli* may represent clonal forms originating from wildlife in the protected upland and wetlands of ANERR. In contrast, MAR profiles of HS isolates showed wide diversity likely related to widespread use of antibiotics in human and domestic animal populations.

The level of antibiotic resistance observed in this study among all HS and NHS isolates (82%) is similar to a previous report for urban and rural waters by Kasper et al. (86). They found 90% of all isolates were resistant to one or more antibiotics. In another study, investigators showed that 80% of strains from municipal waste, river and estuarine water displayed antibiotic resistance (140). Much lower resistance, ranging from 31% to 72%, has been reported for *E. coli* from various aquatic environments (22, 38, 67, 83).

The occurrence of antibiotic resistance among HS *E. coli* is probably due to widespread use of chemotherapeutic drugs, and may reflect the occurrence of plasmid transfer in the alimentary tracts of humans, and in the microbial milieu of sewage systems (57, 67, 68, 150). Several studies have shown that plasmid exchange readily occurs between *E. coli* and other coliform bacteria in stagnant areas of waste water systems (68, 69).

Reported ampicillin resistance among *E. coli* isolates varies from 5.6% in spring water, to 54% for sewage and shellfish (37). In this study, 12.5% of HS and 4.5% of NHS isolates were resistant to ampicillin. One possible explanation for wide variation in ampicillin resistance among reported studies may be due to the composition of bacterial species in different environments, and the exchange of resistance factor (R factors). For example, the species composition of a sample has been shown to be influenced by the frequency, type and proportion of fecal input, and type of recipient water (37, 65). Furthermore, a high proportion of ampicillin resistant *Klebsiella* spp. could transfer R factors to other members of the *Enterobacteriaceae* (10, 11, 99, 110). Therefore, MAR profiles can provide useful information about sources of fecal pollution.

Fatty Acid Methyl Ester Profiles (FAME)

In this study, FAME profiles of HS and NHS *E. coli* isolates were analyzed and compared. Isolates for FAME analysis were selected based on the number of isolates in specific MAR clusters. All isolates contained significant amounts of hexadecanoate (30%), cis 7 hexadecenoate (13%), cycloheptadecanoate (>11%) and summed features 7 (>18%). No significant differences ($P>0.05$) were observed between types and quantities of FAME profiles of HS and NHS isolates (Table 4).

Table 4 Major fatty acids of human source (HS) and nonhuman source (NHS) isolates

Name	Designation[a]	# of isolates		% of fatty acids[b]	
		HS	NHS	HS	NHS
Dodecanoate	$C_{12:0}$	53	51	4	4
Tetradecanoate	$C_{14:0}$	53	51	7	8
Hexadecanoate	$C_{16:0}$	53	51	30	30
Cis 7 hexadecenoate	$C_{16:1}c7$	53	51	13	13
Cycloheptadecanoate	$C_{17:0}cyc$	53	50	11	13
Cyclononadecanoate	$C_{19:0}cyc$	52	47	3	4
Summed feature 3		53	51	8	9
Summed feature 7		53	51	21	18

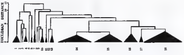
[a] The number before the colon refers to the carbon atoms, the number after the colon refers to the number of double bonds. c, cis; cyc, cyclo

[b] The P-value for HS and NHS isolates was determined by two sided binomial test. For all fatty acids P-value was >0.05

The interrelationships of FAME profiles of *E. coli* from HS and NHS are depicted in the dendrogram (Fig 7), based on the coefficient of similarity generated by the Euclidian distance between pairs of bacteria. On the basis of the MIDI dendrogram software, at a Euclidian distance of about 13.0, all isolates were grouped together as a single cluster, and an approximately 10.0 Euclidian distance formed two major groups, indicating that all isolates were likely to be members of the same species (131). At a Euclidian distance of approximately 4.0, 18 clusters were well separated. Clusters 1, 2, 7, 8, 11, 13 and 17 comprised only HS isolates, whereas clusters 3, 5, 6, 9, 10 and 12 comprised only NHS isolates and clusters 4, 14, 15, 16 and 18 contained isolates from both sources (Table 5). However, the number of isolates in each cluster was not adequate to indicate significance.

Based on less than 10% variance for 85 95% of the FAME profiles, 87 of the 104 isolates were divided into 10 sub groups. It is interesting that subgroup 7, 8 and 10 comprised only HS isolates and sub-group 9 comprised isolates from NHS, whereas the remaining sub-groups comprised isolates from both sources (Table 6). The relationship of the FAME profiles is also illustrated by principal component analysis of the data in two dimensions (Fig 8) and it has been found that HS isolates were more widely distributed throughout the plot compared to NHS isolates. However, no well separated groups for HS and NHS isolates were observed. This suggest a high level of phenotypic diversity within the *E. coli* population. From this study, it has been found that there was

Figure 7 Dendrogram of FAME profiles of human source (HS) and nonhuman source (NHS) *E. coli* isolates. Bottom line indicates number of clusters


EUCLIDEAN DISTANCE
0
5
10
15
14
15
16
17
18

no significant difference between the FAME profiles of HS and NHS of *E. coli* that could be used to predict its source

Table 5. Numbers of human source (HS) and nonhuman source (NHS) isolates in different clusters

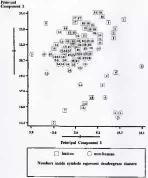
Number of clusters	Number of HS isolates	Number of NHS isolates
1	5	0
2	1	0
3	0	1
4	2	1
5	0	1
6	0	2
7	2	0
8	1	0
9	0	1
10	0	3
11	2	0
12	0	1
13	3	0
14	4	15
15	11	11
16	10	1
17	3	0
18	3	14

Table 8 Numbers of human source (HS) and nonhuman source (NHS) isolates in different sub groups

Number of sub groups	Number of HS isolates	Number of NHS isolates
I	3	9
II	16	5
III	5	8
IV	8	1
V	1	7
VI	2	5
VII	4	0
VIII	1	0
IX	0	9
X	3	0

To our knowledge no study has yet been done to correlate *E coli* FAME profile with sources Fatty acid methyl ester profiles were mainly used for comparative studies at the species level and above for taxonomic possibly systemic investigations. However Harvelaar et al (78) isolated *Aeromonas* strains from patients with diarrhea and from drinking water, analyzed for cell wall FAME profiles and observed that FAME profiles could be used to compare strains from different sources There was little overall similarity between *Aeromonas* strains from human (diarrheal) and from drinking water differences being most pronounced for *A caviae* and least for *A sobria* Kotilainen et al (91) also used this method for identification and typing of coagulase negative

Figure 8 — Two dimensional plot of FAME profiles of *E. coli* isolates generated by principal component analysis


Principal Component 2
25.4
23.8
22.3
20.7
19.1
17.6
16.0
14.4
-9.8
-3.4
3.0
9.3
15.7
22.1
Principal Component 1
□ human
○ non-human
Numbers inside symbols represent dendrogram clusters

Staphylococcus and concluded that coagulase negative *Staphylococcus* can be identified at the species level. Moreover, computerized fatty acid profile correlation analysis can be applied for determining identity, as well as differences between multiple coagulase-negative staphylococcal blood isolates. In contrast, several investigators could not differentiate some pathovars of *Xanthomonas campestries*, *Streptomyces scabies* and members of *Enterobacteriaceae* at the intraspecies level. The treatment of results by principal-component analysis or by other methods of classification did not improve the separation of strains (35, 120, 143). These findings are in agreement with our results. Finally, it can be concluded that fatty acid analysis was unable to differentiate HS and NHS of *E. coli*. However, the usefulness of the MIS as typing tool may be dependent upon the species examined. Some species may exhibit more reproducible and more varied fatty acid profiles than others.

O Serogrouping

A total of 100 isolates (isolates were selected based on the number of isolates in RT clusters) from HS and NHS were tested for O-serogrouping and 77% were typeable. The predominant O serogroups of HS and NHS isolates are shown in Table 7. Human source isolates exhibited 19 serogroups, with 48% belonging to seven serogroups (O2, O20, O28, O79, O148, O153, O159) and the remaining to 12 other serogroups. In contrast, NHS isolates displayed 26 serogroups, with 35% in seven serogroups (O8, O11, Ox13, O19, O150, O152, O15/143), and the remainder in 19 other serogroups.

Thus the seven serogroups most frequently detected in HS isolates were found for only 2 of 50 NHS isolates ($P<0.001$). It was also shown that the majority of serogroups were isolated from HS. Conversely, the majority of NHS serogroups were found in animals such as cattle, chicken, and swine (116). These results were supported by Hartley et al. (74) who compared the prevalent O-serogroups of diseased animals and humans, and found that most strains associated with diarrhea in pigs and humans are different, even though infantile and piglet diarrhea have many common traits. Blanco et al. (27) also found that serogroups from healthy rabbits were generally different from those for *E. coli* associated with disease. The serogroups most frequently detected in diarrheic rabbits were found only in 26% of healthy controls. In contrast, Bettelheim et al. (24) reported that although the distribution of different *E. coli* strains identified by serotype seems to be different in human and animals, many

Table 7 O Serogroups of human source (HS) and nonhuman source (NHS) isolates

O Serogroups	Number (%) of *E. coli* isolates	
	HS (n=50)	NHS (n=50)
2	3(6)	1(2)
8	1(2)	3(6)
11	1(2)	2(4)
x13	0	2(4)
18	0	5(10)
20	2(4)	0
28	2(4)	0
78	7(14)	1(2)
148	3(6)	0
150	0	2(4)
152	0	2(4)
15/143	0	2(4)
153	2(4)	0
159	5(10)	0
Others[a]	10(20)	17(34)
MR	4(8)	0
NT	10(20)	12(24)

[a] 8 and 16 serogroups of HS and NHS isolates, respectively, contained only one isolate are not shown. MR: multiple reaction. NT: nontypeable. x: not accepted by World Health Organization. One NHS isolate was autoagglutinable.

serotypes found in normal humans were also found in other organisms. This situation was illustrated in the analysis of the O:H serotypes of 13,139 strains of *E. coli* isolated from human and 1079 strains isolated from animals, 699 of the latter obtained from cow pats sampled at 22 sites in England and Wales. Typing with a virtually complete set of 154 O and 53 H antisera led to the identification of 709 distinct O:H serotypes, among which 520 were only found in humans, 130 only in animals, and 59 in both. The great diversity of O:H serotypes is illustrated by the finding that, of 79 serotypes identified in cow pat specimens, 65 were found only in one site and 12 were found only at both sites. Moreover, approximately half of the isolates from animals could not be typed in spite of the large number of antisera used.

This study showed that 22% of isolates were nontypeable (Table 7), which is consistent with Blanco's et al. (27) who found that 19% isolates were nontypeable. Moreover, percentage of untypeable isolates from both sources were very similar (20 and 24%, Table 7). This result is supported by other investigators (48) who noted that the frequency of untypeable animal isolates was approximately the same as the frequency of untypeable human isolates obtained in a study of the remote Yanomama Indians of South America.

In conclusion, serogrouping may be a useful tool for differentiating HS and NHS of *E. coli*. Drawbacks include the need for a large bank of antisera, many of which are not commercially available, and that some isolates can not be typed.

Ribotype (RT)

Techniques such as ribotype and pulsed-field gel electrophoresis of chromosomal DNA (5, 138-145) have provided genotypic characterization of bacteria that avoid potential pitfalls associated with variable expression of phenotypic characteristics. Restriction endonuclease analysis of chromosomal DNA has been shown to be useful for identification of many phenotypically indistinguishable bacteria (154). In this study, restriction endonuclease patterns of *E. coli* contained a multitude of fragments, making visual comparison of isolates difficult (Fig. 9A and 10A). Use of a rRNA probe (RT) to highlight restriction fragment length polymorphism (RFLP) yielded hybridization banding patterns that allowed much simpler comparison of isolates (Fig. 9B and 10B). A major advantage of using rRNA gene probes is that the rRNA sequences are highly conserved and are present as multiple copies in bacterial genomes (70). Furthermore, one of the major benefits of using 16S and 23S rRNA for RT *E. coli* is their commercial availability.

In this study, RT profiles of 238 *E. coli* isolates (64 HS, 95 NHS, 30 human and 29 animal feces isolates) were examined. Isolates were selected based on their number in specific MAR clusters. Preliminary experiments of various restriction enzymes (*Hind*III, *Eco*RI, *Sal*I, and *Bgl*I) with a panel of isolates showed that *Hind*III gave the most discriminating profiles of 4 to 13 bands over a size range of 0.7 to 20.0 kilobase pairs (kb) (Fig. 11, 12 and 13). Therefore, all subsequent digestions were performed with *Hind*III.

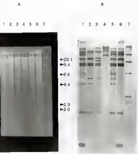


Figure 9 Restriction endonuclease (*Hind*III) digest (A) and representative RTs (B) of human source (HS) *E. coli* isolates. Lanes: 1. SPS 120, 2. SPS 35 3. SPS 65 4. SPS 150 5. SPS 81, 6. *E. coli* ATCC 9637 7. Molecular weight markers in kb

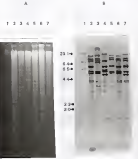


Figure 10 Restriction endonuclease (*Hind*III) digest (A) and representative RTs (B) of nonhuman source (NHS) *E. coli* isolates. Lanes, 1 Molecular weight marker in kb. 2 SP 155, 3 SP 156, 4 SP 157, 5 SP 158, 6 SP 156, 7 SP 162.

Human source isolates showed 41 RTs (Fig. 11 and 14); more than 60% belonged to eleven RTs (Tables 8 and 9, Fig. 14). A binary metric plot of average linkage of HS isolates at an approximately 80% similarity index demonstrated 3 clusters, designated by RP1, RP2 and RP3. Cluster RP1 contained 4 isolates, RP2 contained 3 isolates, and RP3 contained 75 isolates; two isolates were located outside the clusters (Fig. 14). In contrast, NHS isolates exhibited 61 different RTs (Fig. 12 and 15), with 14 RTs were accounting for 50% of isolates (Tables 8 and 9, Fig. 15). A binary metric plot of average linkage of NHS isolates at the same similarity index showed five clusters, e.g. RN1, RN2, RN3, RN4 and RN5; three isolates were located outside the clusters. Clusters RN1, RN2, RN3, RN4 and RN5 contained 34, 2, 49, 2 and 5 isolates, respectively. Within the groups of 84 HS isolates, at 100% similarity level include 64% of isolates, whereas NHS isolates include 50% isolates (Fig 14 and 15, Table 8). A higher degree of homology was also observed for HS isolates at other selected levels of similarity (Tables 8 and 9). At 80% similarity level HS isolates formed 16 RTs, whereas NHS isolates formed 39 RTs. At 100% similarity level 11 separate RTs were obtained for HS isolates (Fig. 14). Each of five RTs contained 2 isolates, one RT 21 isolates, three RTs 3 isolates, and the other two RTs 6 and 9 isolates, respectively. Among NHS isolates at 100% similarity level, 14 RTs were observed. Eight RTs contained the same number of isolates, each of four RTs 3 isolates, one RT 5 isolates, and another RT 18 isolates (Fig. 15).

Table 8 Percentage of human source (HS) and nonhuman source (NHS) *E. coli* isolates at selected similarity indices

Source of isolates	Percentage of isolates at specific similarity indices					
	50%	60%	70%	80%	90%	100%
Human	98	98	96	88	81	64
Nonhuman	98	97	95	88	79	50

Table 9 Number of RTs of human source (HS) and nonhuman source (NHS) *E. coli* isolates at selected similarity indices

Source of isolates	Number of RTs at specific similarity indices					
	50%	60%	70%	80%	90%	100%
Human	3	7	11	14	18	11
Nonhuman	5	9	10	24	39	14

Figure 11 Schematic representation of RTs of human source (HS) *E. coli* isolates obtained by *Hind*III digestion. Prefix SPS indicates HS isolates. Molecular weight markers (kb) are shown on the left.

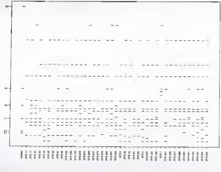

23.1
9.4
6.6
4.4
2.3
2.0
Marker

Figure 12 Schematic representation of RTs of nonhuman source (NHS) *E. coli* isolates obtained by *Hin*fII digestion. Prefix SP indicates NHS isolates. Molecular weight markers (kb) are shown on the left.

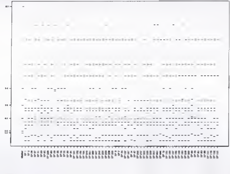

Marker

Figure 13 Schematic representation of RTs of human source (HS) and nonhuman source (NHS) *E. coli* isolates obtained by *Hin*dIII digestion. Molecular weight markers (kb) are shown on the left

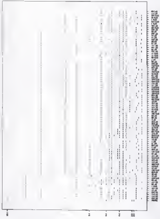


Figure 14 Binary matric plot of average similarity linkage for RT profiles of human source (HS) *E. coli* isolates digested with *Hin*dIII. The similarity index is indicated left the plot

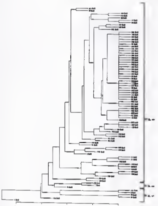


Figure 15 Binary metric plot of average similarity linkage for RT profiles of nonhuman source (NHS) *E. coli* isolates digested with *Hind*III The similarity index is indicated left the plot

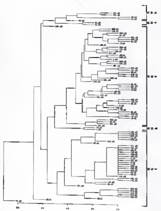
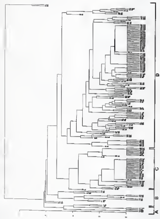


Figure 18 Combined binary metric plot of average similarity linkage for RT profiles of human source (HS) and nonhuman source (NHS) *E. coli* isolates digested with *Hind*III. The similarity index is indicated left the plot


A
B
C
D

Relationship of RT profiles was analyzed for both HS and NHS isolates by combining both databases. Ninety four RTs were identified among 179 isolates, and 24 RTs accounted for more than 60% of the isolates. At a approximately 50% similarity level, four clusters were obtained. Clusters A contained 4 HS and 2 NHS isolates, cluster B 3 HS and 7 NHS isolates, and cluster C most of the NHS isolates, and cluster D most HS isolates. At 100% similarity index, 24 RTs were observed which included 64% HS isolates and 50% NHS isolates. Eight RTs contained only HS isolates and 9 RTs contained only NHS isolates. The remaining RTs contained both HS and NHS isolates (Fig. 16).

Several investigators have used RT to discriminate *E. coli* from different sources (121, 145, 149). Paros et al. (121) described λ-RFLP profiles for *E. coli* O157:H7 isolated from human and bovine feces. They found that 90% of human *E. coli* O157:H7 isolates did not match bovine isolates. Other investigators have demonstrated that RT is a powerful tool for characterizing *E. coli* isolated from specific extra intestinal infections and feces of healthy persons (148). From the present study, it has been found that *E. coli* from HS showed much less diversity in RT profiles than NHS isolates (Fig. 14 and 15). This indicates genetic relatedness among HS, whereas those from NHS isolates were much more genetically diverse. The later situation may result from different *E. coli* populations maintained in more diverse animal reservoirs, such as raccoon, birds, and other animals.

Picard et al. (124) reported that carboxylesterase type B_2 strains separated from B_1 strains by their possession of 6.5 kb, 9.5 kb, 22.4 kb and 3.0 kb, 3.4 kb, 7.0 kb fragments, when digested DNA with *Hind*III and *Eco*RI respectively. However, we did not find any fragment that differentiated HS and NHS of *E. coli*. At a 100% similarity index, HS and NHS isolates showed unique RTs (Fig. 16). These RTs may help to detect the source of pollution in ANERR. In conclusion, RT appears to be useful for genetically distinguishing the HS and NHS of *E. coli*.

Pulsed Field Gel Electrophoresis (PFGE)

Pulsed field gel electrophoresis was performed with *E. coli* isolates with identical MAR and RT profile to determine if they were from the same clonal origin. Human source isolates formed 9 PFGE profiles, three pairs of isolates showed the same MAR, RT and PFGE profiles. However, NHS isolates showed 18 different PFGE profiles. Out of 32 isolates only 4 pairs shared the same MAR, RT, and PFGE profile (Tables 10 and11, Fig. 17), indicating the existence of multiple *E. coli* strains rather then a few single clone in the ANERR. Moreover, there was no differences between PFGE profiles of HS and NHS isolates. Those pairs that shared the same PFGE profile by *SfiI* restriction endonuclease were digested with *NotI* and found that they also showed the identical PFGE profiles (Fig. 18). However, *NotI* digestion profiles were not as distinct as profiles of *SfiI* digestion (Fig. 17 and 18).

Using PFGE, Boerlin et al. (28) did not observe an association between any type of fish product and a particular *Listeria monocytogenes* strains causing human listeriosis. This technique was also used by Edberg et al. (46) to differentiate coliform bacteria from distribution system, source water and clinical isolates. They isolated *Enterobacter cloacae* throughout the water distribution system in New Haven County, CT. From each of the water distribution isolates a single PFGE pattern was generated. However, there was no homogeneity between source and distribution water *E. cloacae* isolates. Moreover, clinical

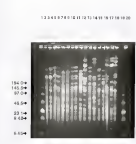


Figure 17 PFGE profiles of human source (HS) and nonhuman source (NHS) *E coli* isolates digested with *Sfi*I restriction endonuclease Lanes 1 and 20 Low range PFG marker in kb, 2 SPS 35 3 SPS 80 4 SPS 42 5 SPS 45 6 SPS 118, 7 SPS 205 8 SPS 486 9 SPS 493,10 SPS 601, 11 SPS 602 12 SPS 561 13 SPS 573 14 SP5, 15 SP 50, 16 SP 93 17 SP 6 18 SP 112

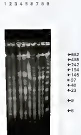


Figure 18 PFGE *NotI* digestion profiles of human source (HS) and nonhuman source (NHS) *E coli* isolates Lanes 1 SPS 118 2 SPS 205 3 SP 14 4 SP 20 5 SPS 42 6 SPS 45 7 SPS 466 8 SPS 493, 9 Lamda ladder PFG marker in kb

Table 10 PFGE, FAME, and O-serogroups of human source (HS) isolates with identical MAR and RT profiles

Strains	MAR	RT	FAME	PFGE	O Serogroup
SPS 35	264	31	ND	D	NEG
SPS 80	264	31	ND	D	NEG
SPS 42	264	2	2	I	MR
SPS 45	264	2	14	I	MR
SPS 118	439	28	ND	I	110
SPS 205	439	28	ND	I	NEG
SPS 466	268	31	7	I	79
SPS 493	268	31	ND	I	NEG
SPS 601	776	10	21	D	85
SPS 602	776	10	ND	D	25
SPS 561	798	31	3	D	79
SPS 573	796	31	ND	D	79

ND Not done, D Different I Identical MR Multiple reaction NEG Negative, i e did not agglutinate with any of the 181 O antisera

Table 11: PFGE, FAME, and O serogroups of nonhuman source (NHS) isolates with identical MAR and RT profiles

Strains	MAR	RT	FAME	PFGE	O Serogroup
SP 5	258	51	ND	D	150
SP 50	258	51	ND	D	NEG
SP 93	258	51	13	D	2
SP 8	256	51	9	D	150
SP 112	256	51	ND	D	15
SP 253	256	51	ND	D	15/143
SP 14	256	59	2	I	19
SP 20	256	59	2	I	19
SP 140	264	54	ND	D	NEG
SP 229	264	54	ND	D	25
SP 233	264	54	ND	D	110
SP 230	264	51	ND	D	NEG
SP 234	264	51	ND	D	NEG
SP 242	264	51	ND	D	8
SP 243	264	51	ND	D	15/143
SP 256	8	44	ND	D	19
SP 328	8	44	ND	D	19
SP 306	0	45	ND	D	152
SP 317	0	45	6	D	NEG
SP 318	0	45	5	D	16

ND: Not done; D: Different; I: Identical; NEG: Negative
i.e. did not agglutinate with any of the 181 O antisera

E. cloacae isolates from patients in the New Haven area showed no identity with *E. cloacae* isolated from water distribution systems. These results are in agreement with our findings because PFGE profiles of HS and NHS did not correlate with source. In contrast, Herbein et al. (77) observed that PFGE can be used to track nonpoint source pollution inputs to tidal waters. Therefore, it can be concluded that PFGE was not a suitable method for differentiating HS and NHS of *E. coli*.

Plasmid Analysis

To determine whether antibiotic resistance was associated with plasmid profiles, 11 isolates were analyzed for plasmids. Their resistant patterns and plasmid profiles are shown in Table 12.

Of 11 isolates, only 5 (45%) were found to harbor one or more plasmids with molecular weights ranging from 2.3 kb to 120 kb. Abimbola et al. (1) examined the plasmid content of 220 antibiotic resistance *E. coli* isolates, and found that 60% harbored plasmids of molecular weights ranging from 0.82 to more than 60 kb. The majority of the plasmids were greater than 2.8 kb. This work supports our study because molecular weight of plasmids were more than 2.3 kb. Isolates resistant to chlortetracycline, sulfathiazole, kanamycin-chlortetracycline-sulfathiazole, neomycin-chlortetracycline penicillin G, oxytetracycline-tetracycline and chlortetracycline-oxytetracycline-sulfathiazole-tetracycline did not harbor detectable plasmids. However, isolates resistance to ampicillin, penicillin G, chlortetracycline nalidixic acid, oxytetracycline-sulfathiazole, and isolates resistant to 9 antibiotics, contained 2, 2, 4, 1, and 7 plasmids respectively. From this experiment, plasmid profiles did not associate with drug resistance. These results are in agreement with those of several investigators (51, 154). However, plasmid profiles and drug resistance have been used to identify and differentiate strains in epidemiological studies, and correlation with drug resistance patterns (50, 154).

Table 12: Plasmid profiles of antibiotic resistant isolates

Resistance patterns[a]	Plasmid size (kb)
A	100, 90
C	None
P	120, 90
Su	None
K-C-Su	None
N-C-P	None
OX-T	None
OX-S	100
C-Na	120, 90, 4.4, 4.0
C-OX-Su-T	None
C-K-Na-N-OX-P-Su-T	14, 10, 8.0, 7.0, 4.0, 3.3, 2.3

[a] A: Ampicillin, C: Chlortetracycline, K: Kanamycin, Na: Nalidixic acid, N: Neomycin, OX: Oxytetracycline, P: Penicillin G, Su: Sulfathiazole, S: Streptomycin, T: Tetracycline.

The number of plasmids per isolate in this study ranged from 1 to 7. There was good agreement with other studies (21, 114) who also reported the same range in plasmid numbers as found in this study. A specific plasmid was not associated with antibiotic resistance. This findings differ from those of Babara et al. (21) who associated drug resistance to 60 MDa plasmid.

Resistance genes may be located on plasmids, chromosomes, or transposons (36). Resistance of *E. coli* isolates to various microbial agents in this study was therefore thought to be mediated by genes located on chromosomes, plasmids or/and on transposons. The lack of relationship between plasmid profile and antimicrobial resistance pattern was not unexpected, because the same antimicrobial resistance pattern can be encoded by unrelated plasmids, transposons, phages and chromosomal genes. Even identical enzyme mediated antibiotic modification has been shown to be encoded by two different genes (104). Antimicrobial resistance may occur due to a spontaneous mutation, or through transmission by other resistant bacteria. Bacteria may also be resistant due to metabolism of the drug, decrease in cell wall permeability, or altering the antibiotic receptor sites. Either of these is usually a result of a mutation (s) in the bacterial genome (41).

Correlation of MAR, FAME, O-Serogroup and RT Profiles and Sources

From the research database, it was found that MAR profile did not correlate with FAME, O-serogroup or RT profile. Moreover, the same RT profile often had a different O serogroup and MAR profile (Tables 9 and 10). This indicates that *E. coli* populations in the ANERR originated from various sources and possibly multiply in the environment. Other investigators (148) also observed that strains with totally different serotypes could have same RT. In contrast, Alos et al. (6) isolated *E. coli* from premature neonates, analyzed for MAR and RT profiles, and observed that specific antibiotic resistance patterns was associated with different RTs. Dalsgaard et al. (40) compared MAR and RT profiles of clinical and environmental *Vibrio cholerae* non-01 strains and observed that *V. cholerae* non 01 strains with the same RT often exhibited similar antibiotic resistance patterns, whereas strains showing the same resistance pattern often had different RTs. Their findings coincide with this study. By combining the data on MAR, FAME, O serogroup and RT profiles, it was also found that only two isolates out of more than 200 *E. coli* from HS and NHS shared the same MAR, FAME, O serogroup, RT, and PFGE profiles (Tables 10 and 11), indicating the existence of heterogenous population of *E. coli* in the ANERR.

This research demonstrated that MAR, O serogroups and RT profiles of *E. coli* correlated with HS and NHS. However, FAME and PFGE profiles did not correlate with source.

Therefore, the next step was to determine if characteristics of HS and NHS *E. coli* isolates correlated with characteristics of isolates obtained directly from human or animal feces. For this purpose, 30 *E. coli* isolates were obtained directly from 3 humans feces. In addition, 29 isolates were obtained from 5 raccoon and an unknown animal feces from the ANERR wildlife reserve. Five isolates were tested per animal, except for one raccoon (4 isolates) for MAR and RT profiles.

MAR studies showed that human and animal fecal isolates had 10 and 5 antibiotic resistance patterns, respectively. The predominant antibiotic resistance pattern for human fecal isolates was chlortetracycline-sulfathiazole (40%), similar to the percentage (33.7%) observed for HS isolates. In contrast 72.4% of animal isolates were sensitive to all antibiotics (Table 2). Human fecal isolates also showed wide variation in MAR profiles compared to animal isolates, corresponding to differences observed for HS and NHS isolates.

From RT experiments, it has been found that RT patterns of human fecal isolates showed high homology with those of HS isolates. In contrast, RT patterns of raccoon isolates varied from raccoon to raccoon, and demonstrated high homology with NHS isolates (Fig. 19, 20 and 21). These data provide evidence that human feces was the probable source of HS isolates collected at the sewage treatment plant and that animal feces constituted the NHS isolates collected in the ANERR.

Figure 18 Binary metric plot of average similarity linkage for RT profiles of human feces *E coli* isolates digested with *Hind*III Prefix H indicates human feces isolates The similarity index is indicated left the plot

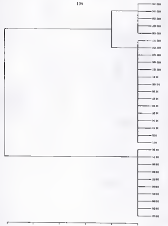


Figure 20 Binary metric plot of average similarity linkage for RT profiles of animal feces isolates digested with *Hind*III Prefix R and A indicate raccoon and unknown animal feces isolates respectively The similarity index is indicated left the plot

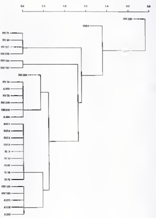
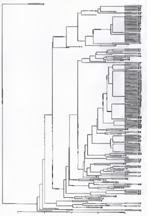

0.0
0.1
0.2
0.3
0.4
0.5
0.6
RW 236
RV14
RV 71
RV 69
RV 111
RW 235
RV 150
RV 167
RW 240
RV 70
A 203
RV 72
RW 209
RW 210
A 204
RV1 1
RV1 2
RV1 3
RV1 5
Ri 9
Ri 10
Ri 30
Ri 34
Ri 76
RV 159
RV 160
A 211
A 212
A 213

Figure 21. Combined binary matric plot of average similarity linkage for RT profiles of human source (HS), nonhuman source (NHS), human and animal faces isolates digested with *Hind*III. The similarity index is indicated left the plot



Validation of MAR and RT Database in RBNERR

It has already been concluded that MAR and RT profiles of HS and NHS *E. coli* isolates correlated significantly with source in the ANERR. However, it was not known if the predictions could be used in another distant estuary. Therefore, to validate the utility of these findings, more than 200 isolates were collected from selected HS and NHS sites in the RBNERR and analyzed for MAR and RT profiles.

Results of MAR experiment showed 25 and 9 antibiotic resistance patterns for HS and NHS isolates, respectively. Like ANERR, HS isolates showed more diversity and higher resistance to single and combinations of antibiotics, than NHS isolates. Among the HS isolates, higher resistance was observed for chlortetracycline-sulfathiazole (25.2%), chlortetracycline-penicillin G-sulfathiazole (26.1%), and chlortetracycline (11.7%), like that in the ANERR. Conversely, 48.1% of NHS isolates were sensitive to all antibiotics. Similar to ANERR, Rookery bay NHS isolates showed greater resistant to chlortetracycline (Tables 2 and 13). The average MAR index for RBNERR's HS and NHS isolates was 0.277 and 0.057, respectively. It was also found that HS isolates of both estuaries showed higher MAR indices than NHS isolates (Tables 3 and 14).

The RT was determined for 50 HS and NHS isolates using *Hind*III restriction endonuclease. Human and nonhuman source isolates showed 19 and 16 RT patterns, respectively (Fig. 22 and 23). Ratio of isolates of RTs of NHS isolates showed similarity between the reserves (1:6). In contrast, ratio of

Table 13 Predominant antibiotic resistance patterns of *E. coli* isolated from human source (HS) and nonhuman source (NHS) in the RBNERR

Antibiotics[a]	Percent with each resistant pattern	
	HS (n = 111)	NHS (n = 104)
C Su	25.2	2.9
C P Su	26.1	ND
C	11.7	18.3
C P	8.3	3.8
Su	ND	18.3
Other resistance patterns[b]	30.7	8.6
Sensitive to all antibiotics	ND	48.1

[a] C: Chlortetracycline, P: Penicillin G, Su: Sulfathiazole; T: Tetracycline; ND: None detected

[b] Each of the other 21 patterns for HS isolates and 4 patterns for NHS isolates contained insignificant number of isolates (not shown)

Table 14 Percentage of RBNERR isolates at different MAR indices

MAR index	HS (%)	NHS (%)
≤0.1	11.7	88.5
0.2	34.2	9.6
≥0.3	54.1	1.9

isolates of RTs of HS isolates was not similar (2.0 and 1.3) (Fig. 14, 15, 22 and 23). These observations may be due to the differences in the sampling sites, i.e. in RBNERR, HS isolates were collected from a creek instead of a sewage treatment plant. In addition, HS site of RBNERR was not typical HS site like ANERR, because this site is influenced by the Manatees, other aquatic animals and birds during the winter season. Therefore, these aquatic animals and birds may contribute *E. coli* population at this site, resulting in more diverse RT profiles compared to those of the HS in ANERR. However, there was no difference between the ratio of RT profiles of NHS isolates in both estuaries, because both were not influenced by HS pollution.

The relationship of RT profiles of HS and NHS *E. coli* isolates was also compared by combining both ANERR and RBNERR databases. Like ANERR, HS and NHS isolates of RBNERR showed specific banding patterns (Fig. 24). It was found that 52% of RBNERR's NHS isolates shared RT profiles with those of ANERR. The unique NHS RT profiles found in both reserves may be due to the presence of different animal populations in each reserve. In addition, 48% of HS isolates shared RT profiles with ANERR isolates (Fig. 25). However, when equal numbers of isolates were compared from both reserves, 64% of HS isolates shared RT profiles. Therefore, it is important to compare equal numbers of isolates from both reserves. Finally, it can be suggested that MAR and RT profiles may provide useful tools for differentiating the level of HS and NHS *E. coli* in estuarine environments.

Figure 22: Binary metric plot of average similarity linkage for RT profiles of RBNERR's human source (HS) isolates digested with *Hind*III. Prefix RP indicates HS isolates. The similarity index is indicated left the plot.

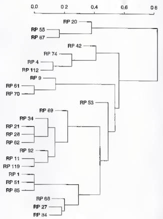

0.0
0.2
0.4
0.6
0.8
RP 20
RP 55
RP 67
RP 42
RP 74
RP 4
RP 112
RP 9
RP 61
RP 70
RP 53
RP 69
RP 34
RP 21
RP 28
RP 62
RP 92
RP 11
RP 119
RP 1
RP 51
RP 85
RP 68
RP 27
RP 84

Figure 23 Binary metric plot of average similarity linkage for RT profiles of RBNERR a nonhuman source (NHS) isolates digested with *Hind*III. Prefix RN indicates NHS isolates. The similarity index is indicated left the plot

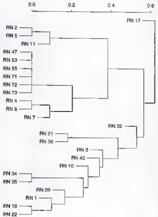

0.0
0.2
0.4
0.6
RN 17
RN 2
RN 5
RN 11
RN 47
RN 53
RN 55
RN 71
RN 72
RN 73
RN 4
RN 8
RN 7
RN 32
RN 21
RN 36
RN 3
RN 40
RN 10
RN 34
RN 35
RN 39
RN 1
RN 18
RN 22

Figure 24 Combined binary metric plot of average similarity linkage for RT profiles of RBNERR's human source (HS) and nonhuman source (NHS) isolates digested with *Hind*III The similarity index is indicated left the plot

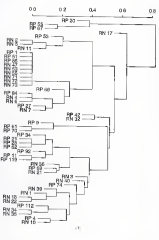
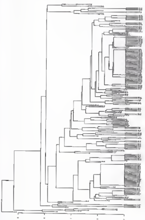

0 0
0 2
0.4
0 6
0 8
RP 20
RP 55
RP 67
RN 17
RP 53
RN 2
RN 5
RN 11
RP 1
RP 51
RP 85
RN 47
RN 53
RN 55
RN 71
RN 72
RN 73
RP 68
RP 84
RN 4
RN 6
RP 27
RN 7
RP 42
RN 32
RP 9
RP 61
RP 70
RP 34
RP 21
RP 28
RP 62
RP 92
RP 11
RP 119
RN 36
RP 69
RN 21
RN 3
RN 40
RP 74
RN 39
RN 1
RN 18
RN 22
RP 112
RN 34
RN 35
RP 4
RN 10

Figure 25 Combined binary metric plot of average similarity linkage for RT profiles of ANERR and RBNERR s human source (HS) and nonhuman source (NHS) isolates digested with *Hind*III The similarity index is indicated left the plot



SUMMARY AND CONCLUSIONS

Fecal pollution can be a major problem in almost all estuaries. This pollution can originate from HS and NHS. Therefore, rapid, sensitive and accurate methods are needed to detect sources of fecal pollution in estuarine environments, thus allowing pollution to be better controlled to provide safe food products.

From this study, it was found that FAME and PFGE profiles of *E. coli* did not correlate with its source. However, MAR, O serogroup, and RT profiles showed a high correlation with source. A main concern over O serogrouping was that 22% of the isolates were nontypeable. In this case, it would be difficult to always draw conclusions about the source (s) of pollution. Moreover, all O antisera are not available to all laboratories.

It is apparent that MAR profiles differed according to the source. Human source isolates showed higher resistance to antibiotics, higher MAR indices, and more diversity than NHS isolates. In addition, predominant antibiotic resistance patterns of HS isolates were different from those of NHS isolates.

There was also significant differences between RT profiles of HS and NHS isolates. Ribotype profiles of HS isolates showed less diversity compared to RT profiles of NHS isolates, indicating greater genetic relatedness among HS isolates supporting the hypothesis that human feces are a main source of human fecal pollution. This evidence was further supported by the results of RT profiles of human fecal isolates. Conversely, RT profiles of NHS isolates showed more diversity and high homology with RT profiles of animal fecal isolates, providing further evidence that the NHS isolates were derived from animal feces in the ANERR.

From the databases established by this research, it was found that MAR, FAME, O-serogroup and RT profiles did not correlate with each other. However, identical RT profiles often exhibited different MAR and O-serogroup. This finding indicates that *E. coli* populations come from a variety of sources and may undergo phenotypic changes in the aquatic environments.

Comparison of MAR and RT profiles in RBNERR suggested the utility of these methods for detecting sources of fecal pollution. However, RT profiles of NHS isolates did show variations likely due to the presence of different animal populations. In such cases, it may be necessary to develop a new database for RT. Further research is needed to associate specific MAR and RT profiles with specific animal species.

Some aquatic environments may be influenced by selective pressure of antibiotics. As a result, specific *E. coli* MAR profiles may not always correlate with HS and NHS of pollution in all estuaries. In these instances, extensive databases may need to be formed to develop association between specific MAR profiles and sources of pollution. However, this problem might be solved by measuring RT profiles because it is a genotypic method not greatly influenced by selective pressure of antibiotics and any other environmental factors. Possibly RT may be more specific than MAR, but in combination they may be very powerful tools. For comparison, the MAR test may be simple for a common laboratory to perform. Both methods can provide resource managers with tools to more accurately manage estuaries and may enhance efforts to improve the natural and commercial quality of estuarine ecosystems, as well as to assess the importance of upstream activities, local storm water runoff and marine activities.

Further research is needed to develop statistical formulas for MAR and RT profiles that can be used to more easily differentiate sources of fecal pollution in estuarine environments. Detecting specific RT banding patterns for HS and NHS isolates may be advantageous in designing primers to develop rapid tests. It is also important to validate these methods in other geographical areas to determine the utility over larger geographical scales.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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